University of Alberta

COMPARATIVE GENETIC ANALYSIS OF PORCINE MYCOPLASMAS

by

Walter Arnold Blank ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of Doctor of Philosophy

in

Microbiology and Biotechnology

Department of Biological Sciences

Edmonton, Alberta

Fall 1999

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For my parents.

ABSTRACT

The members of the class *Mollicutes*, also known as the mycoplasmas, are among the simplest self replicating organisms. These prokaryotes are characterized by the lack of a cell wall, small genomes, and a low G+C DNA content. Three species isolated from the respiratory tracts of pigs, *Mycoplasma hyopneumoniae*, *Mycoplasma flocculare*, and *Mycoplasma hyorhinis* are known to be closely related. In order to determine whether two other porcine mycoplasma, *Mycoplasma hyosynoviae* and *Mycoplasma hyopharyngis*, are also related to these organisms, 16S rRNA phylogenetic studies were performed. It was found that *M. hyosynoviae* and *M. hyopharyngis* were not specifically related either to each other nor to the three other pig mycoplasmas.

Low resolution genetic maps of the genomes of *M. hyopneumoniae*, the causative agent of enzootic pneumonia in pigs (EPP), and *M. flocculare*, a commensal organism, were constructed by pulsed-field electrophoresis (PFGE) and hybridization. It was found that since these species diverged, at least three chromosomal inversions had occurred which differentiate the genomes of these organisms. One of these inversions involved a region in *M. hyopneumoniae* which contains a ciliary adhesin gene (believed to be a pathogenic factor) which is not found in *M. flocculare* and several genes whose products are highly immunogenic. Three different types of repeated elements were also found in *M. hyopneumoniae*, two of which could not be found by hybridization in *M. flocculare* but do not appear to have been involved in the chromosomal rearrangements. The third repeated element, present in one copy in *M. flocculare*, had both of its copies in *M. hyopneumoniae* located near the ends of one of the inverted regions and could potentially have mediated a recombination event.

Assembly of the *M. hyopneumoniae* genomic map was facilitated and complemented by the simultaneous construction of an ordered cosmid library. Five contigs of overlapping clones were assembled, which together represent coverage of approximately 67% of its genome, as calculated by comparing the total sizes of *Eco*RI fragments from the cosmid library to the genome size estimated by PFGE. This library could be useful in discovering genes as either vaccination or therapeutic targets against EPP.

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LIST OF ABBREVIATIONS

A+T	Adenine plus thymine
ATCC	American Type Culture Collection
bp	Base pairs
BSA	Bovine serum albumin
CHEF	Contour-clamped homogenous electrical field electrophoresis
DIG	Digoxygenin
DMSO	Dimethyl sulfoxide
dNTP	Deoxynucleoside triphosphate (N = A, C, G, or T)
EDTA	Ethylene diamine tetraacetic acid
EPP	Enzootic pneumonia in pigs
EtBr	Ethidium bromide
FIGE	Field inversion gel electrophoresis
G+C	Guanine plus cytosine
kb	Kilobase pairs
LB	Luria-Bertani broth
MLO	Mycoplasma-like organism
NEB	New England Biolabs
NTP	Nucleoside triphospate (N = A, C, G, or T)
OD	Optical density
ORF	Open reading frame
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PFGE	Pulsed-field gel electrophoresis
PPLO	Pleuropneumonia-like organism
RE	Restriction endonuclease
rRNA	Ribosomal RNA
SB	Super broth
SDS	Sodium dodecyl sulfate
tRNA	Transfer RNA

1. INTRODUCTION

1.1 The class Mollicutes

1.1.1 Overview

The bacteria belonging to the class *Mollicutes* (Latin, "soft-skinned") are the smallest and simplest of self-replicating microorganisms. (The trivial term "mycoplasma" is commonly used to refer not only to the genus *Mycoplasma* but to all members of this class, and is interchangeable with the term "mollicutes".) The defining characteristic of these bacteria is the lack of a true cell wall – instead, they are bounded by a single lipid bilayer membrane. Typically, mycoplasmas are 0.3-0.8 µm in diameter but are pleomorphic, varying in shape from cocci or bacilli to straight, branched, or (in the case of spiroplasmas) helical filaments up to 40 µm or more in length (Boatman 1973; Boatman 1979; Cole et al. 1973). Colonies have a characteristic "fried egg" appearance when grown on agar and a number of species exhibit gliding motility (Bredt 1973). Mycoplasma genomes have a very low G+C content (under or near 30% for most species) (Herrmann 1992) and range in size from approximately 2 Mb for some spiroplasmas to 580 kb for *Mycoplasma genitalium* – the smallest known bacterial genome (Colman et al. 1990; Fraser et al. 1995; Su and Baseman 1990). A parasitic lifestyle is common among mycoplasmas as a result of limited biosynthetic capabilities due to small genome size. The extreme fastidiousness of mycoplasmas requires the use of complex media components such as animal sera and yeast extract although defined media have been described for several species (Rodwell 1983).

The first mycoplasma cultivated was the causative agent of bovine pleuropneumonia, in the late nineteenth century. For a number of years, mycoplasmas were mistakenly thought of as viruses because their small size allowed them to pass through filters which normally blocked the passage of bacteria (Razin 1992). Later, mycoplasmas were often perceived as stable bacterial L-phase variants, which result from a partial or total loss of the cell wall (Lederberg and Clair 1958) giving a similar morphology. However, by the late 1960's the examination of numerous similarities and dissimilarities between mycoplasmas and L-forms, including cellular and colonial morphology, biochemistry, physiology, pathogenicity, and especially nucleic acid composition (McGee et al. 1967), established the mycoplasmas as a unique subgroup of bacteria.

1.1.2 Taxonomy and Phylogeny

A number of genera comprise the class *Mollicutes*, with distinctions based primarily on morphology, habitat, and nutritional requirements (Razin 1992; Tully et al. 1993; Weisburg et al. 1989). The genus *Mycoplasma* contains the largest number of species, isolated from humans and animals and characterized by a strict need for exogenous sterol for growth. The genus *Ureaplasma* is similar but also has urea as a growth requirement. The genera *Spiroplasma* and *Entomoplasma* , isolated from plants and arthropods, also require sterols but the spiroplasmas have a distinctive helical morphology. Two strictly anaerobic genera, *Anaeroplasma* and *Asteroleplasma*, have been found in the digestive rumen of cattle and sheep, whereas all other mycoplasmas are facultative anaerobes (Razin and Freundt 1984). Neither asteroleplasmas nor the members of the genera *Acholeplasma* and *Mesoplasma*, require sterol supplementation in growth media. In addition, numerous uncultivated and unclassified "mycoplasmalike organisms" (MLOs) and phytoplasmas have also been discovered in association with various host organisms.

The evolutionary origin of the mollicutes proved to be a question of considerable interest, due to their numerous unusual characteristics. Theories ranged from mycoplasmas as present-day relics of a primative ancestor of modern bacteria (Morowitz and Wallace 1973) to degenerative evolution from numerous walled bacteria, suggesting polyphyletic origins (Dienes and Weinberger 1951; Neimark and London 1982). However, advances in phylogenetic analysis and in molecular biology techniques provided the basis for the currently prevailing theory.

The work of Woese and colleagues (Fox et al. 1980; Woese and Fox 1977; Woese et al. 1990) established a new paradigm by dividing the living world into three domains: *Bacteria*, *Archaea*, and *Eucarya*. It was demonstrated that the two groups of prokaryotes, the "true" bacteria and the archaebacteria, were no more closely related to each other than they were to eukaryotic organisms. This was made possible by the comparison of conserved biological macromolecules, in particular the small ribosomal subunit RNA molecule. Although there are different theories and perspectives on the division of the living world e.g. (Gupta 1998; Mayr 1998), that of Woese is most widely accepted within the microbiological community. Differences in sequence between conserved molecules can record the evolutionary history of those molecules (Zuckerkandl and Pauling 1965) and, by corollary, that of the organisms which carry them. This logic can be applied to determine the relationships between individual species.

Comparison of 16S rRNA oligonucleotide catalogs from mycoplasmas belonging to the genera *Acholeplasma*, *Mycoplasma*, and *Spiroplasma* to those of other microorganisms (Woese et al. 1980) showed that they are monophyletic (*i.e.* that all mollicutes have a common ancestor), having arisen from the *Lactobacillus* group of low G+C content Gram-positive eubacteria. Specifically, they are most closely related to a subgroup of clostridia including *Clostridium innocuum* and *C. ramosum*. Analysis of complete 16S rRNA sequences from numerous mycoplasmas and their walled relatives (Weisburg et al. 1989) solidified this relationship and permitted the division of the mollicutes into several distinct groups. The deepest branching (and therefore earliest diverging) among these are the asteroleplasmas, anaeroplasmas, and acholeplasmas. The hominis group contains a number of species isolated from animals including *M. hominis*, *M. lipophilum*, *M. pulmonis*, *M. sualvi*, and *M. neurolyticum*, each with their own distinct cluster within this group. *M. pneumoniae*, *M. muris*, and the ureaplasmas form what is referred to as the pneumoniae group. The final phylogenetically distinct group is comprised of the spiroplasmas, but also contains *M. mycoides* (the type species of the genus *Mycoplasma*), *Mesoplasma florum* and *Entomoplasma ellychniae* (formerly *Acholeplasma florum* and *Mycoplasma ellychniae*) (Tully et al. 1993). It is noteworthy that the genus *Mycoplasma* is polyphyletic in the phylogenetic tree of the mollicutes and the lack of a requirement for exogenous sterol, as evidenced by the mesoplasmas, is not limited to the deepest branches. These examples highlight the difficulty of reconciling a taxonomy based on phenotypic characters with molecular phylogenies (International Committee on Systematic Bacteriology Subcommittee on the Taxonomy of Mollicutes 1990; International Committee on Systematic Bacteriology Subcommittee on the Taxonomy of Mollicutes 1992; Tully et al. 1993).

1.1.3 Evolution of mycoplasmas reflected in physiology and molecular biology

It was evident from the first molecular phylogenetic studies of mycoplasmas that a rapid evolutionary rate is a characteristic of these organisms (Woese et al. 1980). Mycoplasma 16S rRNA molecules are frequently variable in regions normally invariant in other bacteria. This indicates an unusually high mutation rate, since the complicated structure of rRNA often necessitates simultaneous changes in multiple positions, due to base pairing or other physical constraints (Woese 1987; Woese et al. 1985). Mycoplasmas can probably tolerate this higher level of mutation better than most other organisms because their smaller genomes are less likely to incorporate a deleterious mutation in each generation (Maniloff 1992b; Woese 1987). Whether a high mutation rate forced the mycoplasma ancestors to decrease the size of their genomes or if reductions in coding capacity permitted (or perhaps facilitated) less fidelity in DNA replication is not known, but both of these factors have contributed to the unusual biology of these organisms.

Many biological pathways have been lost during the "degenerative" evolution of the mycoplasmas. The lack of a cell wall in all mollicutes indicates that the genetic information required to synthesize this structure was probably lost by the Gram-positive ancestor of this group very early on. Mycoplasmas are parasitic in nature (Razin 1992) and rely on a physically close relationship with their eukaryotic hosts to provide them with biomolecules for which they lack synthetic capability due to genomic reduction. Such nutrients include fatty acids, sterols (except in the case of *Acholeplasma* and *Asteroleplama* species), nucleic acid precursors, amino acids, and vitamins (Rodwell and Mitchell 1979). Energy metabolism is also limited in mycoplasmas due to the lack of cytochromes and a tricarboxylic acid cycle (Pollack 1992), and they rely primarily upon the Embden-Meyerhof-Parnas pathway (although not necessarily glycolysis) for ATP synthesis. Hydrolysis of urea (Romano et al. 1980) and arginine (Barile 1983) are other potential sources of energy for certain mollicutes.

Redundant and (apparently) non-essential genes have also been eliminated from the genomes of mycoplasmas. Genes which are normally present in multiple copies in other organisms, such as rRNAs and tRNAs, are found in limited copy numbers in mycoplasmas (Amikam et al. 1984; Muto et al. 1990). Of the three DNA polymerases present in most bacteria, only Pol III has been detected in mycoplasmas (Fraser et al. 1995; Himmelreich et al. 1996) and numerous other components involved in DNA replication also appear to be missing. Certain types of DNA repair (e.g. light-dependant UV repair, mismatch repair) are also absent from at least some mycoplasmas (Ghosh et al. 1977; Himmelreich et al. 1997), which most likely contributes to the high mutation rate. There is also evidence for multienzyme activity in mycoplasmas whereby a single gene product can perform the function of more than one enzyme. Mycoplasmal lactate dehydrogenase is believed to also be capable of malate dehydrogenase activity because of two additional amino acids in the substrate recognition site of the enzyme (Cordwell et al. 1997). The high frequencies of genomic rearrangement found in antigenic variation systems (discussed below) and the presence of repetitive elements such as insertion sequence-like elements (Bhugra and Dybvig 1993; Zheng and McIntosh 1995) suggest possible mechanisms for recombination-linked loss of genetic material.

In addition to their small size, the genomes of mycoplasmas are distinguished by their unusually low G+C base composition. This has led to one of the most striking features of mycoplasmas, the fact that many species deviate from the universal genetic code. UGA, which is normally a stop codon, is translated as tryptophan in place of UGG (Yamao et al. 1985) in *Mycoplasma*, *Ureaplasma*, and *Spiroplasma* species, but not in the phylogenetically deeper-branching genera. CGG is a nonsense or unassigned codon in some mycoplasma species, although in others it is still read as arginine (Futo et al. 1995a; Oba et al. 1991). Codon usage is strongly biased in mycoplasmas, with a preponderance of codons with A or T in the third position, and even amino acid usage is affected: lysine (coded by AAA or AAG) is used preferentially over arginine (CGN) (Himmelreich et al. 1997; Muto et al. 1984). The AT bias is likely connected to the high mutation rate of mycoplasmas; indeed, a mycoplasma uracil-DNA glycosylase, which repairs spontaneous deamination of cytidine residues, is less efficient than that of other prokaryotes (Williams and Pollack 1990) and could result in a reduced overall G+C content.

1.2 Pathogenicity

Mycoplasmas are known to infect and cause a variety of diseases in humans, animals, insects, and plants (Krause and Taylor-Robinson 1992; Lee et al. 1989; Simecka et al. 1992). The pathogenicity of mycoplasmas cannot be ascribed to any particular virulence determinant but is due to complex multifactorial processes which are not completely understood. Besides their parasitic nature, which puts them in competition with host cells for nutrients the mycoplasmas cannot synthesize themselves, a number of molecular virulence factors have been described for mollicutes.

Colonization and infection of host tissues requires adhesion of mycoplasmas to tissue surfaces. For the most part, mycoplasma species are specific for particular hosts and tissues (Razin 1992), indicating that the adhesion mechanisms in each species are tailored to a particular environment. Sialo-oligosaccharides and glycolipids appear to act as host cell receptors for mycoplasma attachment (Roberts et al. 1989; Zhang et al. 1994) but the mode of attachment is not the same for all mycoplasmas. *M. pneumoniae* and other pathogens such as *M. genitalium, M. gallisepticum*, and *M. pirum* have a specialized terminal structure which gives the cells a flask-shaped appearance (Dybvig and Voelker 1996). A number of adhesion proteins are localized on the surface of this organelle which facilitates attachment by these organisms (Krause 1996). The tip structure is also used by the AIDS-associated mycoplasma *M. penetrans* to invade mammalian cells (Lo 1992). The adhesins found in these species share considerable homology, suggesting a family of host- or tissue-specific cytadherence proteins (Clyde and Hu 1986; Keeler et al. 1996).

Most mycoplasmas, however, do not possess an attachment organelle and rely upon other membrane proteins or lipoproteins for adhesion (Chen et al. 1998; Henrich et al. 1993; Zhang et al. 1995). One such protein, a ciliary adhesin found in *M. hyopneumoniae*, contains two regions of tandemly repeated short sequences of amino acids which vary in size between strains (Hsu et al. 1997; King et al. 1997; Wilton et al. 1998) and may modulate adhesion and provide antigenic diversity. Variable adhesins have also been found in other mycoplasmas (Henrich et al. 1996; Ruland et al. 1994).

Several cytopathic factors have been identified in mycoplasmas (Gabridge et al. 1985) including the polysaccharide portion of a glycoprotein in *M. bovis* which causes inflammation in cattle (Geary et al. 1981) and a cytotoxic 54 kDa membrane protein from *M. hyopneumoniae* (Geary and Walczak 1985). The byproducts of mycoplasma metabolism may also have toxic effects on host cells. In all but the strictly anaerobic mollicutes the superoxide radical is formed as an intermediate during NADH oxidase activity (Pollack 1992). Such reactive oxygen species have been implicated in host cell membrane damage by mycoplasmas (Somerson et al. 1965; Tryon and Baseman 1992). Ureaplasmas produce ammonia during hydrolysis of urea, which may also act to cause local damage to host cells (Ligon and Kenny 1991).

Much of the damage that occurs during mycoplasma infections is not due to the mycoplasmas themselves, but is a result of the host's specific immune response. Lesions in some mycoplasma infections (particularly respiratory infections) are partly composed of infiltrating lymphocytes producing antibodies against mycoplasmas (Howard and Taylor 1985) and it is believed that in chronic mycoplasma-induced arthritides, antigens are retained in cartilage by immune complexes even after the infection has been cleared, resulting in continued inflammation (Cole and Ward 1979). The fact that mycoplasmas can also modulate the immune response is well documented (Cole 1996; Razin et al. 1998; Ruuth and Praz 1989) although it presents another complex picture which is not completely understood. Mycoplasmas are capable of activating lymphocytes in a nonspecific manner, either directly or by inducing cytokines, resulting in proliferation of B and/or T cells, nonspecific antibody production, and inflammatory responses (Razin et al. 1998). The superantigen MAM from *M. arthritidis* (Cole et al. 1996) is of particular interest. It is capable of cross-linking the major histocompatability complex of accessory immune cells with T-cell receptors outside of the conventional antigen binding cleft (i.e. without having undergone antigen processing), triggering cytotoxic T-cell proliferation and autoimmune inflammations such as arthritis. Immunosupression by mycoplasmas may also play a part in the establishment of chronic infections (Razin et al. 1998).

Mycoplasmas must still be able to avoid host defense systems, and despite their limited coding capacity they have developed a number of antigenic variation mechanisms. The variable lipoproteins (Vlps) of *M. hyorhinis* undergo high-frequency size variation (Rosengarten and Wise 1991) as well as exhibiting transcription level phase variation (on/off switching) of multiple Vlp genes (Rosengarten et al. 1993), providing a vast array of surface determinants and ensuring antigenically heterogenous populations within the host. The variable surface antigens in *M. pulmonis*, which are somehow associated with a complex restriction and modification system (Bhugra et al. 1995; Dybvig et al. 1998), and apparent gene conversion ability in *M. pneumoniae* adhesion proteins (Ruland et al. 1994) further demonstrate how mycoplasmas are capable of evading the immune system and adapting to changing environments.

1.3 Porcine mycoplasmas

1.3.1 *Mycoplasma hyopneumoniae*

M. hyopneumoniae was isolated in 1965 as the causative agent of enzootic porcine pneumonia (EPP) (Goodwin et al. 1965; Mare and Switzer 1965), a highly transmissible chronic disease which is a worldwide problem for the pork industry. EPP is associated with lowered weight gain and poor feed efficiency (Pointon et al. 1985) as well as increased susceptibility to secondary infections (Ciprian et al. 1988), resulting in economic losses estimated at $200 million annually in the US alone (Hsu and Minion 1998). *M. hyopneumoniae* has also been isolated from cases of arthritis in grower pigs (Kobisch and Friis 1996) and is the primary isolate from fibrinous pericarditis lesions in slaughter pigs, suggesting a causative role (Buttenschøn et al. 1997).

The *M. hyopneumoniae* bacterium itself has an oval or round morphology ranging in diameter from 0.2-0.7 µm. It can be cultivated in liquid media (Friis 1975) but is extremely slow growing. Glucose is oxidized during growth and hexokinase activity is present, indicating a glycolytic pathway (Jensen et al. 1978). Its circular genome has been measured at 1070 kb (Huang and Stemke 1992; Robertson et al. 1990) and has a G+C content which has been measured variously at 33% (Stemke et al. 1985) and 27.5% (Kirchhoff and Flossdorf 1987). Phylogenetic analysis of its 16S rRNA sequence (Stemke et al. 1992; Weisburg et al. 1989) places *M. hyopneumoniae* in the *M. neurolyticum* cluster of the hominis group, closely related to *M. hyorhinis* and *M. flocculare*, two species also found in the respiratory tracts of swine.

Infection of swine by *M. hyopneumoniae* appears to proceed from colonization of the trachea and bronchi. Scanning electron microscopy (Kobisch and Friis 1996; Mebus and Underdahl 1977) of these structures shows a close association of *M. hyopneumoniae* with ciliated epithelial cells within 1-2 weeks. As the infection progresses, a gradual loss of cilia is observed with mycoplasmas accumulating over the remaining ciliated cells. This clearing coincides with macroscopic lesions of the lungs, particularly in the apical and cardiac lobes. *In vitro* studies indicate that *M. hyopneumoniae* is capable of adhering to red blood cells (Young et al. 1989) and tissue culture monolayers (Zielinski et al. 1990), and a 54 kDa protein isolated from *M. hyopneumoniae* membranes is capable of inducing a cytopathic effect on tissue culture monolayers (Geary and Walczak 1985). A thin-layer chromatography overlay assay indicates that *M. hyopneumoniae* binds specifically to sulfatide, globoside, and monosialoganglioside GM3 glycolipid receptors (Zhang et al. 1994). As is common among many mycoplasmas, *M. hyopneumoniae* is capable of stimulating macrophages to secrete the proinflammatory cytokines IL-1, IL-6, and TNF-α (Asai et al. 1993).

Beta-lactams and other antimicrobial agents which target bacterial cell walls are obviously ineffective when dealing with mycoplasma infections. *M. hyopneumoniae* has been shown to be susceptible to a variety of antibiotics *in vitro* (Hannan et al. 1997; ter Laak et al. 1991), but this does not necessarily correspond to *in vivo* activity. Tiamulin has been used in the treatment of EPP although its efficacy is questionable (Ross and Cox 1988). Fluoroquinolones have been reported to be effective against *M. hyopneumoniae* infections (Ross 1992).

Currently available vaccines based on killed *M. hyopneumoniae* cells, cell extracts, or membrane fractions have given mixed results (Berner 1995; Dohoo and Montgomery 1996; Kobisch et al. 1987; Kristensen et al. 1981; Ross et al. 1984), usually reducing lung pathology in subsequently infected pigs but not preventing colonization of *M. hyopneumoniae*. Numerous antigenic proteins have been characterized in

M. hyopneumoniae, including a lactate dehydrogenase (Haldimann et al. 1993), a ribonucleotide reductase (NrdF) (Fagan et al. 1996), a heat shock protein (Chou et al. 1997), lipoproteins (Futo et al. 1995a; Wise and Kim 1987), the previously mentioned ciliary adhesin (King et al. 1997; Wilton et al. 1998; Zhang et al. 1995), and a newly identified cytadherent glycoprotein (Chen et al. 1998). These molecules are being evaluated for their efficacy as vaccines, some in combination with novel antigen delivery systems. For example, a recombinant NrdF antigen has been expressed in the attenuated *Salmonella typhimurium aroA* strain (Fagan et al. 1997). Inoculation of mice with this live oral vaccine elicits a significant lung mucosal IgA response against NrdF, although it has yet to be tested in pigs.

1.3.2 *Mycoplasma flocculare*

Mycoplasma flocculare is also an inhabitant of the porcine respiratory tract. First isolated and described in 1972 (Friis 1972; Meyling and Friis 1972), *M. flocculare* is very similar to *M. hyopneumoniae*, sharing growth characteristics and antigenic cross-reactivity (Bölske et al. 1987; Freeman et al. 1984; Jensen et al. 1978; Ro and Ross 1983; Stemke and Robertson 1990). *M. flocculare* is, however, a distinctly separate species although 16S rRNA studies indicate it is the closest relative of *M. hyopneumoniae* (Rose et al. 1979; Stemke et al. 1992). Its genome of 890 kb (Huang and Stemke 1992; Robertson et al. 1990) has a similar G + C content to that of *M. hyopneumoniae* but shares only about 10% DNA/DNA hybridization (Stemke et al. 1985) and is not methylated at GATC or CTCGAG sequences, in contrast to *M. hyopneumoniae* (Chan and Ross 1984; Frey et al. 1992). Most importantly, *M. flocculare* does not appear to be pathogenic (Armstrong et al. 1987; Strasser et al. 1992) although experimental infection of pigs can induce histological changes (Friis 1973).

1.3.3 *Mycoplasma hyorhinis*

M. hyorhinis is commonly found in the nasal cavaties of healthy pigs. Although in most cases of infection clinical disease does not occur, stress or other illness may precipitate a septicemic *M. hyorhinis* infection in young pigs resulting in polyserositis and arthritis (Ross 1992). During the acute stages of the disease serosal membranes throughout the body cavity become inflamed. After approximately two weeks the disease shifts into a subacute phase, involving joint inflammation and lameness, and the organisms may be isolated from synovial fluid for up to six months after infection.

M. hyorhinis is also a significant contaminant of laboratory tissue cultures (McGarrity and Kotani 1985). Such infections present considerable problems in research involving cell cultures by having often unpredictable effects upon the cultures in terms of growth characteristics, metabolism, virus propagation, immunological response, and so forth.

1.3.4 *Mycoplasma hyosynoviae* and *Mycoplasma hyopharyngis*

Mycoplasma hyosynoviae is an arginine-utilizing, non-glucose fermenting mycoplasma which is responsible for arthritis in 40-100 kg (i.e., 3-6 month old) swine (Ross and Duncan 1970; Ross and Karmon 1970). This disease is characterized by sudden acute lameness accompanied by swollen joints with an increased volume of synovial fluid, from which the organism can be isolated. *M. hyosynoviae* is also commonly isolated from the upper respiratory tracts of both unaffected and arthritic pigs and can persist in the tonsils of adult swine (Kobisch and Friis 1996).

Mycoplasma hyopharyngis is a relatively uncharacterized species of mycoplasma also isolated from the upper respiratory tracts of swine (Erickson et al. 1986). Like *M. hyosynoviae*, it can metabolize arginine but not glucose, but in contrast to

M. hyosynoviae it produces phosphatase and is serologically distinct from that and other mycoplasma species. It is not known whether *M. hyopharyngis* is pathogenic, as it is generally isolated from healthy pigs although it has been found in arthritic joints (Kobisch and Friis 1996).

1.4 Bacterial genomic mapping

The typical bacterial genome consists of a single circular DNA chromosome, but the last decade has shown that bacterial genomes can be more complex. In 1989 it was reported that *Rhodobacter sphaeroides* contains two separate circular chromosomes (Suwanto and Kaplan 1989) and that the spirochete *Borrelia burgdorferi* has a linear chromosome (Baril et al. 1989; Ferdows and Barbour 1989). Since then linear chromosomes have also been found in *Streptomyces* species (Leblond et al. 1996; Lin et al. 1993) and *Rhodococcus fascians* (Crespi et al. 1992). Numerous bacterial species, particularly members of the alpha subgroup of the proteobacteria, have multiple chromosomes which are circular, or in the case of *Agrobacterium tumefaciens*, one circular and one linear chromosome (Jumas-Bilak et al. 1998).

Bacterial genomics, the study of bacterial chromosomes and their genes, has grown into a full fledged science over the last decade. By determining the arrangement of genes in diverse prokaryotes, chromosome structure in a higher "architectural" sense and how it relates to genetic function may be studied, as well as setting the stage for comparative genomics. Comparison of the genetic maps of closely related bacteria, on the other hand, can give insight into their recent evolutionary history (and the [chromosomal] mechanisms thereof) and provide clues about phenotypic differences, laying the groundwork for more detailed work.

The tools for investigating bacterial genomes have advanced rapidly, allowing nearly any organism to be studied. The remainder of this section will examine these tools and how they have been applied, particularly to the study of mycoplasma genomes.

1.4.1 Genetic linkage mapping

The earliest bacterial genomic maps were constructed by measuring linkage (recombination frequency) between genes through the experimental transfer of DNA into organisms, either by conjugation and interrupted mating or using phage-mediated transduction. Linkage maps have been determined for a number of well-characterized bacteria, including *Escherichia coli* (Bachmann 1990) and *Bacillus subtilis* (Anagnostopoulos et al. 1993). However, construction of linkage maps is restricted to those organisms for which the appropriate genetic tools are available (thereby excluding the vast majority of bacteria, including mycoplasmas) and the labour-intensive nature of this mapping method also limits its usefulness.

1.4.2 Ordered clone maps

Molecular cloning techniques facilitate the construction of libraries of genomic DNA which can be propagated in a heterologous host such as *E. coli*, permitting the genomic analyis of organisms for which traditional genetic tools have not been developed. Overlapping clones can be ordered into contigs which comprise a physical map of the genome, which may then be analyzed in more detail since the genetic material is readily amplified.

A variety of cloning vectors can be used to construct such maps. A phage λ clone library was used to map the *E. coli* W3110 chromosome (Kohara et al. 1987). Overlaps were detected by comparing the restriction maps of several thousand clone inserts and assembled into a nearly complete map consisting of several hundred clones. Relatively small insert size, requiring analysis of a large number of clones, is a major drawback of λ libraries, but they are capable of carrying genetically unstable sequences and genes which are toxic to the *E. coli* host.

A cosmid vector was used to clone the genome of *Mycoplasma pneumoniae* by chromosome walking, using the ends of the cosmid inserts as probes (Wenzel and Herrmann 1988; Wenzel and Herrmann 1989). Only 34 overlapping cosmids were required to cover the approximately 800 kb genome, except for one unclonable gap which was closed with two λ clones and a plasmid. The 578 kb genome of the closely related *M. genitalium* was cloned with 20 cosmids and a λ clone (Lucier et al. 1994). Cosmids are capable of carrying inserts large enough to reduce the number of clones required to cover bactrial genomes while still being easily mapped themselves. However, they are prone to deletions due to recombination and cannot carry certain unstable genomic fragments (as seen in the mycoplasma maps).

Other cloning vectors capable of carrying large genomic fragments, such as yeast artificial chromosomes (Kuspa et al. 1989) and F-plasmid based bacterial artificial chromosomes (Brosch et al. 1998) have also proven to be useful, particularly for bacteria with large genomes.

1.4.3 Pulsed-field gel electrophoresis

1.4.3.1 Description of PFGE

In conventional gel electrophoresis, DNA molecules migrate through a gel matrix in the direction of an electric field and are separated by size due to the sieving effect of the gel. Smaller DNA molecules can travel further through the gel because they can pass through most of the pores in the matrix, allowing them to move toward the anode in a fairly direct manner. Larger molecules, however, require pores which will accomodate their larger size and must therefore take a more indirect route through the gel matrix, resulting in a lower overall mobility. Thus, for a particular gel concentration there is a range of DNA sizes which may be separated, below which the smaller DNA molecules are no longer hindered by sieving and above which the larger molecules elongate, align themselves along the electric field and travel in an end-on fashion known as "reptation" (Fangman 1978) through the gel. These reptating molecules all travel with the same mobility, regardless of size. Under normal electrophoretic conditions, DNA molecules larger than approximately 20 kb can no longer be effectively separated. Low gel concentrations (as low as 0.1% agarose) and the use of low voltage gradients help in the separation of larger fragments but present difficulties in the handling of gels and require very long run times.

Pulsed-field gel electrophoresis (PFGE) (Schwartz and Cantor 1984) takes advantage of the alignment of reptating DNA molecules with the electric field. If the field is replaced with a second electrical field at a different angle, the molecule has to reorient itself in the new direction before it can begin to migrate through the gel again. The time taken by a molecule to realign is dependent on its length, so smaller molecules may begin to migrate while the larger ones are still being reoriented. Constant switching between two electrical fields can therefore separate large DNA molecules with the path of mobility through the gel actually being the vector sum of many zig-zag steps. Through pulsed-field electrophoresis, DNA molecules of up to megabase lengths may be separated, as Schwartz and Cantor (1984) demonstrated with the chromosomes of *Saccharomyces cerevisiae*.

Although all pulsed-field separations operate on the same basic principles, refinements to the configurations of PFGE systems has greatly improved the reproducibility and usefulness of the technique. The earliest PFGE systems (Carle and

Olson 1984; Schwartz and Cantor 1984) made use of non-homogenous electric fields, which were thought to be required for proper separations but prevented molecules from travelling in straight paths (since the strength of the field varied across the gel), making size estimations and lane comparisons difficult. Demonstration that homogenous electric fields are also effective at separating large molecules (Carle et al. 1986; Chu et al. 1986; Gardiner et al. 1986; Southern et al. 1987) has led to modern systems which make exclusive use of homogenous fields.

Field-inversion gel electrophoresis (FIGE) (Carle et al. 1986) is the simplest of these, in which a standard electrophoresis apparatus is used with a switching device which reverses the direction of the electric field. If the switch intervals in the forward direction are of longer duration than those in the reverse direction, the net migration of the molecules will be in a straight path forward. It has, however, been demonstrated that this migration is not necessarily a function of size, especially near the upper limit of resolution for a particular set of running conditions (Heller and Pohl 1989; Lalande et al. 1988). Although this band scrambling can occur in all forms of PFGE and even in conventional electrophoresis, it is particularly pronounced in FIGE. Ramping of switch times during the run (Heller and Pohl 1990) and modulation of the reverse direction voltage gradient, in a method known as zero-integrated field electrophoresis (Turmel et al. 1990), help to alleviate this problem.

PFGE systems in which the alternating electric fields are at obtuse angles to each other (as opposed to the 180° reorientation angle of FIGE) are in very common use today. This can be accomplished by mechanical rotation of either the gel or the electric field (Southern et al. 1987), or by contour-clamped homogenous field electrophoresis (CHEF), in which multiple electrodes surrounding the gel in a hexagonal array are electronically controlled to produce a constant voltage gradient across the gel (Chu et al. 1986). These methods permit the separation of a large number of DNA samples reproducibly over a relatively short period of time. A reorientation angle of greater than 90° is necessary for proper separation of large molecules (Chu et al. 1986; Southern et al. 1987), and although many systems are set at a fixed angle of 120°, others are programmable over a range of angles to optimize separation of particularly large DNAs.

Of equal importance with the electrophoretic principles behind PFGE is the preparation of the high molecular weight DNA required for such separations. Normal preparation of genomic DNA in solution exposes the molecules to shear forces which physically reduce the length of the DNA. Entire chromosomes are prepared by suspending cells or protoplasts in molten agarose, allowing solidification in molds, then digesting cellular materials *in situ*, leaving intact DNA molecules, which are protected from shearing by being immobilized in agarose (Birren and Lai 1993; Schwartz and Cantor 1984). The DNA thus embedded in agarose blocks can be further enzymatically manipulated as necessary.

1.4.3.2 PFGE mapping

Pulsed-field techniques have proven to be particularly valuable for the physical mapping of bacterial chromosomes. Digestion of agarose-embedded DNA with infrequently cutting restriction endonucleases (REs) results in a limited number of large restriction fragments which can be separated by PFGE. The fragments can then be reassembled into a low-resolution restriction map of the bacterial chromosome by a number of methods: individual fragments may be eluted, labelled, and hybridized to gel blots of other digests to identify overlapping fragments (Lee et al. 1989; Pyle and Finch 1988); partial digests, making use of specific DNA methylases (Hanish and McClelland

1990); two-dimensional electrophoresis methods which make use of partial digests in the first dimension and completed digests in the second or complete digests with two REs (Bautsch 1994); hybridization with "linking clones" (cloned genomic DNAs which contain rare restriction sites) to identify adjacent fragments (Kieser et al. 1992); as well as combinations of these and other methods. Gene markers can be placed upon the resulting maps by hybridization of probes to gel blots. However, because of the large size of the restriction fragments these loci are approximate, and the specific order of closely linked genes can often not be determined unless they are separated by the recognition sequence of one of the REs used.

The first microbial genome map constructed by PFGE was that of *E. coli*, both making use of and filling gaps in the Kohara ordered clone library (Smith et al. 1987). Since then a vast array of bacterial genomes have been physically mapped by PFGE. Mollicutes genomes are particularly amenable to this type of analysis because of their low G+C content (which REs with GC-rich recognition sites cut infrequently) and small size: *M. mycoides* (Pyle and Finch 1988; Pyle et al. 1990), *M. mobile* (Bautsch 1988), *U. urealyticum* (Cocks et al. 1989), *M. pneumoniae* (Wenzel and Herrmann 1989)*M. genitalium* (Colman et al. 1990), *M. capricolum* (Miyata et al. 1991), *M. hominis* (Ladefoged and Christiansen 1992), *M. hyopneumoniae* and *M. flocculare* (Huang and Stemke 1992), *S. citri* (Ye et al. 1992), *M. gallisepticum* (Tigges and Minion 1994), *S. melliferum* (Ye et al. 1994a), and *M. gallisepticum* (Gorton et al. 1995) have all been characterized in varying degrees of detail. In addition, PFGE has been used to determine the genome sizes of numerous mycoplasmas (Neimark and Lange 1990; Pyle et al. 1988; Robertson et al. 1990).

Comparison of genomic arrangements between mycoplasmas is difficult because each mapping project makes use of different genetic markers, but some comparative investigations have been performed. A study involving five strains of *M. mycoides* and the related *Mycoplasma* strain PG50 (Pyle et al. 1990) showed that four small colony variant *M. mycoides* strains had highly conserved genomes with respect to both gene order and restriction fragment size. A large colony variant was very similar with respect to gene order, although it appears as though an inversion of approximately 140 kb has occurred. The map of strain PG50 suggests that it has undergone at least two more genomic rearrangements and has very little conservation of restriction sites, indicative of the high point mutation rate in mycoplasmas. Mapping of five strains of *M. hominis* (Ladefoged and Christiansen 1992) demonstrated a high degree of gene order conservation (although a 300 kb inversion was noted in one strain) despite the fact that the strains were heterogenous in terms of serology, SDS-PAGE protein analysis, and nucleic acid hybridization. Two spiroplasma species appear to differ in their genomic arrangement by a large inversion involving nearly half of the chromosome (Ye et al. 1994a).

1.4.4 Genome sequencing

DNA sequencing technology and computer analysis techniques have advanced far enough to permit the highest degree of genomic analysis available – determination of the complete genetic code of an organism. Since the sequencing of the bacterium *Haemophilus influenzae* was completed in 1995 (Fleischmann et al. 1995), over a dozen complete genomes have been published, from each of the bacterial, archaeal, and eukaryotic domains, and more are constantly being added to their number. The immense amount of data generated by sequencing projects provides an unprecedented opportunity for macromolecule analysis and for comparisons between organisms.

Among the published sequences are those of *M. genitalium* and *M. pneumoniae*. The 580,070 base pairs which make up the *M. genitalium* genome were sequenced using a shotgun strategy (Fraser et al. 1995). Nearly 4 Mb of sequence data from either end of approximately 5000 random plasmid clones, along with previously obtained random sequences (Peterson et al. 1993), was assembled by computer into contigs and incorporated into the *M. genitalium* physical map (Lucier et al. 1994). The remaining short gaps were filled by identifying and sequencing templates which spanned them. Analysis of the sequence indicated a total of 470 open reading frames (ORFs) which were predicted to code for proteins, 374 of which were putatively identified by showing homology to sequences in protein databases.

In contrast to the random approach used to sequence *M. genitalium*, the 816,394 base pairs in the *M. pneumoniae* genome (Himmelreich et al. 1996) were determined by a directed strategy based upon the ordered cosmid library (Wenzel and Herrmann 1989). Subclones from this library were sequenced by primer walking, allowing both strands of the genome to be sequenced with much lower redundancy than *M. genitalium* but requiring the synthesis of over 5000 oligonucleotide primers. 677 ORFs were predicted although less than half could have functions assigned to them by database homologies.

Regardless of the sequencing methodology used, the complete sequences of the two mycoplasmas presents a unique opportunity to compare these closely related organisms. A systematic comparison of the genomes was performed by the group which sequenced the *M. pneumoniae* genome (Himmelreich et al. 1997). The most striking observation made was that all of the ORFs predicted in the *M. genitalium* sequence are present in *M. pneumoniae*. Identified functions among the *M. pneumoniae*-specific genes were a restriction-modification system, two specific phosphotransferase systems, an alcohol dehydrogenase, and the complete arginine dihydrolase pathway. Gene order between the two species was highly conserved within six genomic subdivisions, although the order of these segments themselves differed. Interestingly, each of these six regions was flanked by repetitive elements, indicating that the chromosomal rearrangements had occurred by homologous recombination (Himmelreich et al. 1997).

Mycoplasma genomes have long been thought of as ideal candidates for complete sequencing (Morowitz 1984), not only because their small sizes are particularly suited to such a task, but also for the insight which could be gained into what constitutes an autonomously-replicating living system. An attempt to define the minimum number of genes required for life was made by comparing the first two sequenced genomes, those of *H. influenzae* and *M. genitalium* (Mushegian and Koonin 1996). After correcting for redundant and parasite-specific genes and accounting for non-orthologous genes which perform the same function, a set of 256 genes common to both organisms was identified as being close to what the authors thought of as the minimal amount of information required to sustain a modern-type cell. However, this analysis failed to account for major differences between *H. influenzae* and *M. genitalium*, for instance, a possible cytoskeleton in *M. genitalium* (Krause 1996) to compensate for the lack of a cell wall in this organism (Himmelreich et al. 1997). As well, it still remains to be seen if other essential (but non-orthologous) proteins are encoded by genes to which no function has yet been assigned – or for that matter, whether the correct function was assigned in the first place (Pollack 1997).

1.5 Research Objectives

The mycoplasmas *M. hyopneumoniae*, *M. flocculare*, and *M. hyorhinis* are all common isolates from the porcine respiratory tract. All three are more closely related to one another than to other mycoplasmas (Stemke et al. 1992) and are therefore likely to be descendants from a single species inhabiting the respiratory tract of pigs or some porcine ancestor. One of the goals of my research was to determine whether two other porcine respiratory tract inhabitants, *M. hyosynoviae* and *M. hyopharyngis*, belong to the same phylogenetic clade as the other "pig mycoplasmas" by analysis of their 16S rRNA sequences. This would indicate whether the porcine host had been colonized by mycoplasmas on more than one occasion or if the original colonizing species evolved to fill different niches within the same host. This may give insight into the host specificity of mycoplasmas and how easily they may cross species boundaries.

A previous study had created PFGE physical maps of the genomes of *M. hyopneumoniae* ATCC 25095 and *M. flocculare* (Huang and Stemke 1992) and placed genetic markers for the separated 5S rRNA and 16S-23S rRNA loci (Taschke et al. 1986). Another goal of my research was to create a map of *M. hyopneumoniae* strain J (the type strain of the species, as opposed to strain ATCC 25095) and place genetic markers on the maps of both species, allowing comparison of the overall structure of their chromosomes. Because the genome of *M. hyopneumoniae* is approximately 200 kb larger than that of *M. flocculare* (Herrmann 1992; Huang and Stemke 1992) it is reasonable to believe that the pathogenicity of *M. hyopneumoniae* is facilitated by genes not present in the *M. flocculare* genome. Comparative mapping could help identify chromosomal rearrangements and deletions which may include genes with potential as therapeutic or vaccination targets.

In conjunction with PFGE, another approach to mapping the *M. hyopneumoniae* genome is the creation of an ordered cosmid library. Not only would such a map be of a higher resolution, but genes and other regions of interest identified by comparative mapping could be subcloned and analyzed in further detail. A cloned *M. hyopneumoniae* genome could also act as the basis for a directed strategy genome sequencing project similar to that of *M. pneumoniae* (Hilbert et al. 1996; Himmelreich et al. 1996).

2. MATERIALS AND METHODS

2.1 Chemicals and enzymes

Chemicals were purchased from Anachemia Canada (Montreal, PQ), BDH Inc. (Toronto, ON), Boehringer-Mannheim Canada (Laval, PQ), Eastman-Kodak (Rochester, NY), Fisher Scientific (Fair Lawn, NJ), J. T. Baker (Phillipsburg, NJ), Life Technologies (Gaithersburg, MD), and Sigma (St. Louis, MO). Nylon membranes were purchased from Amersham Canada (Oakville, ON) and Boehringer-Mannheim. Radiolabelled nucleotides were purchased from DuPont-NEN (Boston, MA) and Amersham. X-ray film was from Eastman-Kodak and Fuji (Stamford, CT), and instant photographic film was from Polaroid (Cambridge, MA).

Bacterial growth media components were obtained from Difco (Detroit, MI) except for animal sera (Gibco, Grand Island, NY and Flow Laboratories, McLean, VA) and fresh yeast extract, which was prepared by technical staff in our lab.

Restriction endonucleases (Table 2.1) and other enzymes used in this study were purchased from Boehringer-Mannheim, Life Technologies, New England Biolabs (Beverly, MA), Pharmacia (Piscataway, NJ), or Promega (Madison, WI).

DNA oligonucleotides (Table 2.2) were kindly provided by Dr. F. Laigret (INRA, Bordeaux, France) or synthesized by Dr. K. L. Roy (Dept. of Biological Sciences, University of Alberta). Random hexamers were from Boehringer-Mannheim.

Table 2-1 Restriction enzymes used in this study

Enzyme	Recognition sequence	Supplier
Alu I	`AG/CT`	Boehringer-Mannheim
Apa I	`GGGCC/C`	Boehringer-Mannheim
*Apa*L I	`G/TGCAC`	NEB
*Asp*718 I	`G/GTACC`	Boehringer-Mannheim
*Bam*HI	`G/GATCC`	Promega
*Bgl*I	`GCCNNNN/NGGC`	Pharmacia
Dra I	`TTT/AAA`	Pharmacia
*Eco*R I	`G/AATTC`	Boehringer-Mannheim
*Hin*d III	`A/AGCTT`	Boehringer-Mannheim/ BRL
*Ngo*MI	`G/CCGGC`	Promega
*Not*I	`GC/GGCCGC`	Boehringer-Mannheim
*Pst*I	`CTGCA/G`	Boehringer-Mannheim
Pvu II	`CAG/CTG`	Boehringer-Mannheim
Sac I	`GAGCT/C`	Boehringer-Mannheim
*Sac*II	`CCGC/GG`	Pharmacia
Sal I	`G/TCGAC`	Boehringer-Mannheim
*Sau*3A I	`/GATC`	Boehringer-Mannheim
*Sma*I	`CCC/GGG`	Pharmacia

Table 2-1 Continued

Enzyme	Recognition sequence	Supplier
Ssp I	`AAT/ATT`	NEB
Xba I	`T/CTAGA`	Boehringer-Mannheim
*Xho*I	`C/TCGAG`	Pharmacia

Table 2-2 Oligonucleotide primers used in this study. Degenerate bases are indicated in parentheses, I denotes inosine.

Name	Sequence (5'-3')	Source or reference
-40 Forward	GTTTTCCCAGTCACGACGTTGTA	Amersham/USB
-50 Reverse	TTGTGAGCGGATAACAATTTC	Amersham/USB
rRNA A	G (AT) ATTACCGCGGC (GT) GCTG	(Lane et al. 1985)
rRNA B	CCGTCAATTC (AC) TTT (AG) AGTTT	(Lane et al. 1985)
GWS 6	TCTCAGTTCGGATTGA	G. W. Stemke
GWS 35	GGAGCAAATAGGATTAG	G. W. Stemke
GWS 104	CCGACCATTGTAGCACG	This study
GWS 105	TTATTAAGTCTGGAGTC	This study
GWS 106	GTGAGGTAATGGCCCAC	This study
GWS 107	GTTTGGTCAAGTCCTGC	This study
GWS 118	ACGGGATAGTATTTAGTTTACTA	G. W. Stemke
GWS 119	ATTCAAAGGAGCCTTCAAGCTTCA	G. W. Stemke
GWS 120	TCTTCCCTTACAACAGCAGTTTACA	G. W. Stemke
DnaA I	TATAATCC (TA) TT (AG) TTTAT (TA) TATGG	F. Laigret
DnaA II	(AT) AC (AT) GT (AT) GTATGATC (TC) C (TG) (AT) CC (AT) CC	F. Laigret
Ung I	ATTTTAGG (ATG) CAAGA (TC) CCATA (CT) CAT	F. Laigret
Ung II	GTATTTAAATAATAAT (TA) AC (CAT) CC	F. Laigret

Table 2-2 continued

Name	Sequence (5'-3')	Source or reference
ValS I	CC (AT) CC (AT) CC (AT) AA (TC) GT (AGT) AC (AT) GG	F. Laigret
ValS II	CA (AT) GC (AT) GGAAT (ATG) C (TG) ATG (ACT) C C	F. Laigret
GltS I	GC (AT) CC (AT) (TAG) C (AT) CC (AT) AG (AT) GG	F. Laigret
GltS II	(AT) GCTTT (AT) GG (TC) AA (GA) TA (TCA) CCC	F. Laigret
TrpS I	TG (CT) ATTGT (ATG) GATCAACATGC	F. Laigret
TrpS II	TGATCTTC (ACT) CC (ACT) AC (AT) GG (CAT) AC	F. Laigret
RpoB I	AA (TC) ATGCAAGG (ACGT) CA	F. Laigret
RpoB II	TCTTC (AG) AA (AG) TT (AG) TA (ACT) CC	F. Laigret
RpoD I	GG (AT) (TC) T (AT) ATGAA (AG) GC (AT) GT	F. Laigret
RpoD II	G (AT) AC (CT) AC (CT) TA (AGT) (GT) C (ACGT) G T (TC) CG	F. Laigret
P1	AGAGTTTGATCCTGGCTCAGGA	(Robertson et al. 1993)
P6	GGTAGGGATACCTTGTTACGACT	(Robertson et al. 1993)
NrdF L	TTTGCCGAACCTTTTTACA	(Fagan et al. 1996)
NrdF R	AATCAGCAATAAAATTTAGC	(Fagan et al. 1996)
GyrA L	GG (GATC) AA (GA) TA (CT) CA (CT) CC (GATC) CA (CT) GG	(Mizrahi et al. 1993)

Table 2-2 continued

Name	Sequence (5'-3')	Source or reference
GyrA R	AT(GA)TT(GCAT)GT(CGAT)GCCAT(CGAT)CC(CGAT)AC	(Mizrahi et al. 1993)
Adh L	TCAGTATTCATTTGAAGCTA	(Wilton et al. 1998)
Adh R	CTCGATTAGTTCAACCTCTG	(Wilton et al. 1998)
LDH L	GTGCTGGAAATGTCGGAAAT	(Haldimann et al. 1993)
LDH R	ATAAGCAACAAAAGATGAAT	(Haldimann et al. 1993)
HSP60 L	GAATTCGAIIIIGCIGGIGA(TC)GGIACIACIAC	(Goh et al. 1996)
HSP60 R	CGCGGGATCC(TC) (TG)I(TC) (TG)ITCICC(AG)AAICCIGGIGC	(Goh et al. 1996)
Pyk 1	ATGGT(AGCT)GC(AGCT)CG(ACT)GG(AGCT)GA	(Gubler et al. 1994)
Pyk 2	TC(AGCT)AGCAT(CT)TC(AGCT)GT(AGCT)GC	(Gubler et al. 1994)
P46 3'	GGAAGCTGCAGTTAACTTTTAGAAATTTTAGGCATC	(Futo et al. 1995a)
P46 5'	GGAAGGGATCCACTTCAGATTCTAAACCACAAGCCGA	(Futo et al. 1995a)
RecA 3'	TT(TC)AT(ACT)GA(TC)GC(ACGT)GA(GA)CA(TC)GC	(Dybvig et al. 1992)
RecA 5'	TT(TC)AT(ACT)GA(TC)GC(ACGT)GA(GA)CA(TC)GC	(Dybvig et al. 1992)

2.2 Bacterial strains and cloning vectors

Mycoplasma hyopneumoniae strain J and *M. flocculare* ATCC 27716 were kindly provided by Dr. J. Robertson (Medical Microbiology and Immunology, University of Alberta), and *M. hyopneumoniae* ATCC 25095 was provided by Dr. Y. Huang (Department of Microbiology, University of Alberta). *M. hyosynoviae* strain S-16 was a gift from Dr. J. Tully (NIAID, Frederick Maryland). *Escherichia coli* XL-1 Blue MRF' supercompetent cells were purchased from Stratragene (LaJolla, CA) and *E. coli* strain HB101 was provided by Dr. B. Leskiw (Dept. of Biological Sciences, University of Alberta).

Plasmid vectors pBluescript SK+ and pCRScript were purchased from Stratragene. The cosmid vector pcosRW2 (Wenzel and Herrmann 1988) was a gift from Dr. R. Herrmann (University of Heidelberg, Heidelberg, Germany). Plasmid clones of the *Mycoplasma mycoides* glycine tRNA and elongation factor Tu genes were a gift from Dr. L. Finch (University of Melbourne, Parkville, Australia). An M13 mp19 clone containing the *M. flocculare* 5S rRNA gene was provided by Dr. Y. Huang. A number of plasmid clones of random fragments of *M. hyopneumoniae* genomic DNA were constructed by Dr. G.W. Stemke and partially sequenced by P. Ostashewski.

2.3 Media and growth of bacteria

2.3.1 *M. hyopneumoniae* and *M. flocculare*

Both *M. hyopneumoniae* and *M. flocculare* were grown in a "pig broth" based on Friis' formulation (Friis 1975) consisting of Hank's balanced salts solution (Sigma) (30% v/v), Brain Heart Infusion (0.49% w/v), PPLO Broth (0.52% w/v), horse serum (10% v/v), pig serum (10% v/v), fresh yeast extract (0.9% v/v), and phenol red (13 mg/L), pH

7.5. Several 2 mL cultures grown at 37 °C in late logarithmic phase (as indicated by colour change of phenol red) were used to inoculate 20 mL of medium each. When these had grown, they were pooled and 40 mL were used to inoculate 400 mL of broth. When the pH of the cultures had reached 6.8 (corresponding to 10^9-10^{10} colour changing units/mL by serial dilution of 0.2 mL of culture in 1.8 mL of medium), the cells were harvested by centrifugation at 33 000 × g, 4 °C for 35 minutes. The cell pellet was washed with 1% (w/v) NaCl solution. Stocks were made by addition of sterile glycerol to cultures to a final concentration of 15% and frozen at -70 °C.

2.3.2 *M. hyosynoviae*

M. hyosynoviae was grown in a broth (HSI) based on ATCC Medium 243 (Cote 1992) (2.5% (w/v) Heart Infusion Broth, 10% (v/v) fresh yeast extract, 20% (v/v) horse serum, 50 mg/L ampicillin) (83% (v/v) final concentration) supplemented with pig serum (to 7.5% v/v), mucin (10 g/L), celite (1 g/L), L-arginine (0.5 g/L), and bromthymol blue (13 mg/L), pH 7.0. A growing 1.5 mL culture was used to inoculate 60 mL of HSI. The culture was incubated at 37 °C without shaking until an alkaline shift was noted, as indicated by colour change of the bromthymol blue. Two 475 mL bottles of HSI were then inoculated with 25 mL of culture each and incubated until the medium pH had risen to 7.8. Cultures were harvested as above.

2.3.3 *Escherichia coli*

E. coli cultures were grown in Luria-Bertani (LB) broth (10 g/L NaCl, 10 g/L tryptone, 5 g/L yeast extract), or Super Broth (SB) (5 g/L NaCl, 12 g/L tryptone, 20 g/L yeast extract) with shaking at 37 °C. Solid growth media contained1.3% agar. Where necessary, 10 mg/L tetracycline and/or 50 mg/L ampicillin was added to selective media.

Stocks were made by the addition of sterile 40% glycerol to an equal volume of culture, freezing on dry ice and storage at -70 °C.

2.4 Isolation of DNA

2.4.1 Genomic

High molecular weight genomic DNA for construction of genomic libraries was prepared by resuspending the mycoplasma cell pellet in 8 mL of 50 mM Tris pH 7.5, 50 mM EDTA, addition of 1 mg proteinase K, and lysed at 50 °C with 2 mL 1% sodium dodecyl sulfate (SDS) for 3 hours. The DNA solution was gently extracted with phenol, phenol:chloroform, and chloroform, then transferred to prewetted sterile dialysis tubing (Arthur H. Thomas Co., Philadelphia PA) and equilibrated against two changes of 1 L TE at 4 °C for 8 hours each. The DNA solution volume was reduced to approximately 2 mL by laying the dialysis tubing on a bed of dry polyethylene glycol (PEG), transferred to sterile tubes, and stored at 4 °C.

Mycoplasma genomic DNA was isolated by resuspending the cell pellet from 800 mL of culture in 4 mL TE (10 mM Tris pH 8, 1 mM EDTA) and cells were lysed with 1 mL 10% SDS at 40 °C for 1 hour. Proteinase K (1 mg; Boehringer-Mannheim) was added and the mixture was incubated for another 3 hours. The mixture was extracted twice with an equal volume of phenol, twice with phenol:chloroform (1:1) and twice with chloroform. DNA was precipitated with the addition of sodium acetate to 0.3 M and 2 volumes of 95% ethanol, pelleted at 7 500 × g at 4 °C for 10 minutes, washed with 70% ethanol, redissolved in 2 mL TE, and stored at 4 °C. DNA concentrations were determined on a fluorimeter with the dye H33258 (Hoechst).

2.4.2 Plasmid/cosmid

Supercoiled plasmid or cosmid DNA was isolated using a modified alkaline lysis procedure. For minipreps, 1.5 mL of overnight *E. coli* culture was pelleted and the cells were resuspended in 150 µL of 0.3 M sucrose, 25 mM EDTA, 25 mM Tris, pH 7.5. The cells were lysed with 150 µL 0.3 M NaOH, 1% SDS until the mixture was viscous and clear, then neutralized with 200 µL 3 M potassium acetate, pH 5.5, on ice. Tubes were centrifuged at 12 000 × g for 10 minutes, the pellet of bacterial debris was removed with a toothpick, and the supernatant was extracted with 500 µL of phenol:chloroform (1:1). The aqueous phase was removed to a fresh tube and DNA was precipitated with 1 mL of 95% ethanol. After centrifugation, the pellet was washed with 70% ethanol, dried, dissolved in 50 µL TE or mQ water, and stored at -20 °C. Larger DNA preparations were scaled up appropriately, although cultures of 500 mL or more were processed using a DNA maxiprep kit (Promega).

2.5 Preparation of DNA for CHEF

Washed cell pellets from 800 mL of mycoplasma culture were resuspended in 1-2 mL of TE. An equal volume of low melting temperature agarose (1.5% w/v in TE, cooled to 50 °C) was added, the mixture was pipetted into 2×5×10 mm casting molds and refrigerated. Once solidified, the agarose blocks containing mycoplasma cells were added to ten volumes of digestion buffer, containing 0.45 M EDTA, 10 mM Tris pH 7.5, 1% sodium laurylsarcosine, and 0.1 mg/mL proteinase K. Digestion buffer was changed three times, each time allowing overnight incubation at 50 °C. The blocks were then washed and stored in a solution of 50 mM EDTA and 50 mM Tris pH 7.5 at 4 °C.

Before digestion, the DNA blocks were equilibrated in 100 volumes of sterile mQ water. The blocks were cut into 1×2×5 or 2×2×5 mm blocks, equilibrated in five volumes of the appropriate restriction endonuclease buffer with 0.1 µg/mL bovine serum albumin (BSA), and digested with 5-10 units/100 µL of restriction enzyme overnight at the recommended temperature.

2.6 Gel electrophoresis

2.6.1 Agarose gel electrophoresis

Routine DNA electrophoresis was done with agarose gels in 1× TAE (40 mM Tris-acetate, 1 mM EDTA). If ethidium bromide (EtBr) was not included in the running buffer (at 25 µg/L), gels were stained in a 100 µg/L EtBr solution with gentle agitation for 15 minutes followed by destaining in distilled water. Gels were viewed on a UV transilluminator and either photographed with Polaroid 665 high-speed instant film or recorded with a digital imaging system (Appligene-ONCOR, Gaithersburg, MD) and saved to diskette. Subsequent analysis was performed on a Macintosh computer using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/).

2.6.2 CHEF

CHEF gels were 1% agarose in 0.5× TBE (45 mM Tris-borate, 1 mM EDTA). Agarose blocks containing digested DNA and molecular size markers (phage lambda DNA concatamers (Promega and New England Biolabs) or PFGE Midrange Marker I (New England Biolabs)) were immobilized in the wells with molten agarose. Electrophoresis was performed in a DR-II CHEF apparatus (Bio-Rad, Cambridge, MA) at 165 V, 13 °C, for 40-50 hours. Switch intervals were usually 1-13 s or 2-15 s for gels to be used for Southern blotting, although times were altered to permit better separation of particular fragments. Gels were stained for at least 2 h in 100 µg/L EtBr, destained for a similar period of time, then photographed under UV light with Polaroid 663 positive/negative instant film or recorded with the digital imager as above.

2.7 Purifying DNA fragments from agarose

Large DNA fragments were purified from CHEF gels using the DNAMax kit (Life Technologies). Briefly, a block of agarose containing the fragment of interest was excised from the gel and melted in a solution of 3 M NaI. A powdered glass matrix was added to bind the DNA, pelleted, washed several times with a high salt/ethanol solution, and the DNA was eluted with TE or mQ water.

Smaller DNA fragments, such as PCR products or smaller (<2 kb) restriction fragments were electroeluted from the gel (Zhen and Swank 1993). Gels were stained with EtBr in the running buffer, and a well was cut directly in front of the band of interest. The well was filled with 1X TAE plus PEG and EtBr, and the electrical current was reapplied to the gel until the band had completely run into the well. The solution containing the DNA was then pipetted into a microcentrifuge tube, extracted with phenol/chloroform, the DNA was precipitated with 95% ethanol and redissolved in TE.

2.8 Southern blotting

After photography, CHEF gels were exposed to ultraviolet light in a Bio-Rad UV chamber (nicking program) to introduce breaks in the high molecular weight DNA. Otherwise, DNA was briefly depurinated in 0.25 M HCl. DNA was then denatured by shaking the gels gently in 1.5 M NaCl, 0.5 M NaOH (2.5 gel volumes) for 15 minutes. Gels were equilibrated in transfer buffer (1.5 M ammonium acetate, 0.1 M NaOH, 2.5 volumes) while the downward capillary transfer setup (Chomczynski 1992) was assembled. After transfer for 2 to 16 hours, the position of the wells was marked with pencil on the membrane prior to removing the gel. The membrane was washed in denaturing buffer, neutralized in 1 M Tris (pH 7.5), rinsed in distilled water, and the DNA was fixed to the membrane by UV crosslinking or drying at 80 °C.

2.9 PCR

Polymerase chain reactions were performed in mixtures containing either a PCR base buffer of 10 mM Tris pH 8.8, 5 mM KCl, 10 ng/µL BSA, and 0.5 mM $MgCl_2$, or with PCR buffer (Boehringer-Mannheim). Magnesium ion concentrations were optimized between 0.5-7.5 mM for each primer/template set. Oligonucleotide primers were analysed for self-complementarity and optimal annealing temperature using the computer program Oligo 4.0 (National Biosciences, Plymouth, MN) and were present in the reactions at a concentration of 1 µM for degenerate primers and 0.1 µM for specific primers. Template DNA was present at a final concentration of 0.2 ng/µL and the reaction was catalyzed by Taq DNA polymerase (0.5-1 µL/reaction) which had been prepared by technical staff in our lab. Reactions were carried out either in a volume of 100 µL on a PHC-2 thermal cycler (Techne, Princeton, NJ) or in 50 µL on a PE 2400 PCR cycler (Perkin-Elmer, Foster City, CA). Cycling conditions varied with the particular product being amplified. If specific primers were being used, the cycling program consisted of template denaturation at 94 °C, primer annealing at 5 °C below the determined optimum temperature, and product extension at 72 °C (1 minute for each step) repeated thirty times. For degenerate primers, the first 5 annealing steps were carried out at 37 °C with the remaining steps at 5 °C below the determined optimum temperature. Alternatively, a "touchdown" program (Roux 1994) was used, in which the annealing temperature was decreased by 1 °C each cycle for the first 15 cycles (beginning at 50 °C) followed by annealing at 55 °C for a further 20 cycles. PCR products were analyzed on 1.0-1.5% agarose gels and stained with ethidium bromide after electrophoresis. If a particular PCR product needed to be purified from nonspecific amplification products, the band corresponding to the desired product was stabbed with a cut off micropitettor tip and the resulting agarose plug was added to 10 µL mQ water, frozen, and thawed while being centrifuged at 12 000 × g. 1 µL of the supernatant was used as template in a second round of PCR amplification under the same conditions as the first round.

PCR products were cloned using the pCRScript cloning kit (Stratagene) and transformed into *E. coli* XL1-Blue MRF' supercompetent cells (Stratagene) according to the manufacturer's directions.

2.10 DNA sequencing

The *M. hyosynoviae* 16S rRNA gene was sequenced using the Sequenase 7-deaza-dGTP kit (Amersham/USB) with α-^{33}P-dATP. Single stranded plasmid DNA was obtained by denaturing 10 µg of plasmid in a volume of 50 µL with 3 µL 2 M NaOH at room temperature for 10 minutes and desalting through a Sepharose CL-48 spun column (Pharmacia). After primer annealing, the sequencing reactions were carried out according to kit instructions or in the presence of 10% dimethyl sulfoxide. Reaction products were separated on 6% polyacrylamide TBE gels in the presence of formamide and urea. After fixing and drying, the gels were exposed to X-ray film overnight.

PCR products were sequenced on an ABI 373 automated DNA sequencer using dye terminator chemistry by Molecular Biology Services, Department of Biological Sciences, University of Alberta.

2.11 Sequence analysis

DNA sequences were edited and assembled using programs from the GCG suite (Genetics Computer Group, Madison, WI). Sequences were compared against public databases at the National Center for Biotechnology Information network server (http://www.ncbi.nlm.nih.gov/) using the BLAST program (Altschul et al. 1990).

Evolutionary analyses were performed on 16S rRNA sequences either by subjecting multiple sequence alignments (generated by GCG) to maximum parsimony analysis with bootstrapping using programs from PHYLIP (Felsenstein 1993) or by submitting sequences directly to the ribosomal RNA database project (Maidak et al. 1994) for maximum likelihood analysis. Phylogenetic trees were made graphically using TREEVIEW (Page 1996).

2.12 Cosmid library construction

High molecular weight mycoplasma DNA (25 μg) was subjected to partial digestion with *Eco*RI (1 unit/μg DNA) in the presence of BSA for 30-40 minutes at 37 °C and the reaction was stopped with the addition of EDTA to 50 mM. DNA fragments were separated by centrifugation (SW-40Ti rotor, 25 000 rpm, 16h at 4 °C) on a 12 mL sucrose density gradient (15-40% w/v in TE). 0.5 mL fractions were collected and aliquots were analysed on a 0.3% agarose gel. Those fractions containing DNA fragments 30-50 kb long were pooled. The DNA was precipitated, redissolved, treated with alkaline phosphatase (Boehringer-Mannheim), extracted with phenol/chloroform and re-precipitated. The cosmid vector pcosRW2 was prepared by digestion with *Pvu*II, treatment with alkaline phosphatase, and extraction with phenol/chloroform, then digestion with *Eco*RI, resulting in two arms of the cosmid vector, each with a phage lambda *cos* site. Vector DNA thus prepared (4 μg) was ligated to 1 μg of partially digested mycoplasma

DNA overnight at 14 °C with 2 units of T4 DNA ligase (Boehringer-Mannheim) in a volume of 20 μL.

Ligated DNA (1 μg) was packaged in phage lambda packaging extracts (Gigapack, Stratagene) according to the manufacturer's directions. Packaged cosmids were then transfected into *E. coli* (strain HB101 for *M. flocculare* cosmids, XL-1 Blue MRF' for *M. hyopneumoniae*) by addition of 100 μL of packaging reaction diluted 1:10 in SM buffer (O.1 M NaCl, 8 mM $MgCl_2$, 50 mM Tris pH 7.5, 0.01% (w/v) gelatin) to 100 μL of cells (OD_{600}=0.5 in 10 mM $MgSO_4$) from an overnight culture grown in LB supplemented with 2 g/L maltose and 10mM $MgSO_4$. The mixture was incubated at room temperature for 30 minutes, 800 μL LB was added and incubated at 37 °C for 1 hour to allow for antibiotic resistance expression, and the transfected cells were plated on LB agar containing ampicillin and tetracycline and grown overnight. Individual colonies were grown in SB containing ampicillin and tetracycline and cosmids were purified as described above. Restriction analysis was performed by digesting 5 μL (0.5-1 μg) cosmid DNA with 2-5 units of *Eco*RI in the presence of ribonuclease A (0.5 μg/μL).

2.13 Colony blots

Individual cosmid-containing colonies were picked with a toothpick and patched onto LB plates containing antibiotics in an ordered grid pattern (up to 100 colonies per plate). The plates were incubated at 37 °C until colonies were visible (4-8 hours) and the cosmid DNA was affixed to nylon membranes as described by Sambrook et al. (1989). Nylon membrane discs (82 mm diameter) were laid on the agar surface and holes were pierced through the membrane into the agar to allow for later realignment. The membranes were then processed by laying them, colony side up, on sheets of blotting paper soaked with the following solutions for 5 minutes each, in succession: 10% (w/v) SDS; 1.5 M

NaCl, 0.5 M NaOH; 1 M Tris, pH 7.5, 1.5 M NaCl; and 2× SSC (1× SSC contains 150 mM NaCl, 15 mM sodium citrate pH 7.0). The membranes were allowed to dry at room temperature and the DNA was crosslinked to the membrane with UV light. Prior to hybridization, the membranes were gently scrubbed in a solution containing 1% SDS and 5X SSC in order to remove bacterial debris.

2.14 Probe synthesis

2.14.1 Random-primed DNA labelling

Double-stranded DNA fragments and single-stranded M13 DNA were radioactively labelled using the method of Feinberg and Vogelstein (1983). DNA (100-500 ng) was mixed in a total volume of 15 μL with 5 μL 4X NT buffer (200 mM Tris pH 7.8, 20 mM $MgCl_2$, 10 mM dithiothreitol, 200 μg/mL BSA) and 1 μL random hexamers (50 A_{260} units/mL), boiled for 5 minutes, and incubated at 37 °C for 15 minutes. α-^{32}P-dATP or -dCTP (10-20 μCi) was added, along with 2 μL deoxyribonucleotide mixture (containing 1 mM each remaining dNTP) and 1-2 units Klenow DNA polymerase (Boehringer-Mannheim). Reactions were incubated at 37 °C for 2-16 hours.

2.14.2 Oligonucleotide labelling

Oligonucleotides were 5' end-labelled in a 10 μL reaction mixture containing 200 ng oligonucleotide, 20 μCi γ-^{32}P-dATP, 1X kinase buffer, and 1 unit T4 polynucleotide kinase (Pharmacia). Reactions were incubated at 37 °C for 1 hour.

2.14.3 Riboprobe synthesis

Cosmid templates were prepared for transcription by digesting two aliquots of each cosmid separately with *Hin*dIII and *Xba*I, inactivating the enzymes at 65 °C for 30 minutes, then pooling the two digests. The DNA was precipitated with 95% ethanol and redissolved to approximately 1 µg/µL in mQ water. Plasmid templates were linearized by digestion with *Sac*I. Digoxygenin (DIG) labelled RNA probes were synthesized in a 10 µL reaction mixture containing 1 µg DNA template, 1 µL ribonucleotide mixture (10 mM each of ATP, CTP, and GTP; 6.5 mM UTP; and 3.5 mM DIG-11-UTP (Boehringer-Mannhiem)), and 5 units of either T7 RNA polymerase (New England Biolabs or Boehringer-Mannhiem) or SP6 RNA polymerase (Life Technologies or Boehringer-Mannhiem), all in the transcription buffer provided by the enzyme manufacturer. Transcriptions were carried out at 37 °C for 1-3 hours and were terminated by the addition of 10 µL of formamide and stored at -20 °C.

2.15 Hybridization

2.15.1 Southern & colony blots

Hybridization solution for use with DNA probes consisted of 6X SSPE (1X SSPE is 150 mM NaCl, 10 mM NaH_2PO_4, 1.25 mM EDTA, pH 7.4), 5X Denhardt's reagent (0.5% (w/v) each of Ficoll 400 (Pharmacia), polyvinylpyrrolidone, and bovine serum albumin), 0.5% SDS, and 100 µg/mL denatured salmon sperm DNA. A solution containing 50% (v/v) formamide, 5X SSC, 1% (w/v) Blocking Reagent (Boehringer-Mannheim), 1% (w/v) SDS was used for RNA probes. Nylon membranes were blocked with hybridization solution (1 mL/10 cm^2) for 1-2 hours in heat sealed plastic bags, followed by replacement with fresh hybridization solution and addition of the probe. Double-stranded DNA probes were first denatured at 95 °C for 5 minutes. Intraspecies hybridization (*M. hyopneumoniae-M. hyopneumoniae* or *M. flocculare-M. flocculare*) was carried out at 65 °C (50 °C if the hybridization solution contained formamide) whereas interspecies hybridization (*M. hyopneumoniae-M. flocculare*) was done at 37 °C. Hybridizations were allowed to proceed overnight. Hybridization solutions containing DIG labelled probes were stored at -20 °C for re-use. Membranes were washed at room temperature with 2X SSPE or SSC, 0.1% SDS, and if high stringency was required, at 65 °C with 0.5X SSPE or SSC, 0.1% SDS. Radiolabelled probes were detected by wrapping the membrane in plastic wrap and exposing to X-ray film at -70 °C for up to two weeks depending on signal strength. DIG-labelled probes were detected by chemiluminescence using CSPD (Boehringer-Mannheim) after immunological detection with anti-DIG–alkaline phosphatase conjugates (Boehringer-Mannheim) according to manufacturer's protocols with some modifications (Engler-Blum et al. 1993).

2.15.2 Cross hybridization

Cosmid clones were analyzed for overlaps using the cross-screening procedure (Locke et al. 1996). Briefly, target DNA from up to 45 cosmids clones (0.3 µg each) was applied to nylon membranes in a series of parallel stripes using a Miniblotter 45 Channel System (Immunetics, Cambridge, MA). After fixing the DNA and prehybridizing the membrane in 50% formamide hybridization solution, the membrane was replaced in the Miniblotter apparatus one quarter-turn from its original orientation. DIG labelled RNA probes were then applied in another series of stripes perpendicular to the target DNA, such that each probe had the opportunity to hybridize with each target. Amount of probe used varied with the particular template and RNA polymerase used to synthesize the probe, usually 2-5 µL of each terminated T7 labelling reaction and twice as much for those synthesized with SP6 RNA polymerase. Hybridizations were carried out at 50 °C for 2 hours. Membranes were washed at high stringency and probes were detected by chemiluminescence or with the colorimetric reagents NBT and X-phosphate (Boehringer-Mannheim) by following the manufacturer's instructions.

2.15.3 Stripping membranes

Radiolabelled DNA probes were stripped from membranes by boiling in 0.1% (w/v) SDS for 15 minutes and allowing the solution to cool to room temperature. Removal of probe was monitored by use of a Geiger counter or exposure to X-ray film. If necessary, the boiling procedure was repeated. DIG-labelled RNA probes were hydrolized by washing the membranes twice in 0.2 M NaOH, 0.1% SDS at 37 °C for 15 minutes. After stripping membranes were stored either dried at room temperature or at 4 °C in 2X SSPE. The nylon membranes could be reprobed up to 10 times.

3. RESULTS

3.1 The *M. hyosynoviae* 16S rRNA gene

3.1.1 PCR and Sequencing

Genomic DNA extracted from a culture of *M. hyosynoviae* was used as template for the PCR amplification of the 16S rRNA gene. The primers P1 and P6, which are specific for 5' and 3' ends of the 16S rRNA genes of Gram-positive organisms and members of the class *Mollicutes* (Robertson et al. 1993), were used to amplify a DNA fragment appoximately 1.5 kb in length (Figure 3-1). The PCR products were blunt-end cloned into the plasmid vector pCRScript and subsequently transformed into *E. coli*. Two plasmid clones (pR1 and pR3) obtained from separate PCR reactions were chosen for sequencing.

Both strands of the pR3 insert were sequenced according to the strategy outlined in Figure 3-2. Sequence data obtained from the M13 forward and reverse primers and from the conserved 16S rRNA primers A and B (Lane et al. 1985) was used to select the previously synthesized primers GWS 6 and GWS 35, and primers GWS 104-107 were designed to complete sequencing of both strands. One strand of the pR1 insert was sequenced and this was compared to that of pR3. Five point differences were seen between the sequences of pR1 and pR3. When the sequences were aligned with the 16S rRNA sequences of other mollicutes, the variations were seen to be in conserved regions, and one of the clones always had the agreeing nucleotide, which was taken to be correct. The 1499 bp sequence of the amplified 16S rRNA gene fragment (Figure 3-3) was submitted to GenBank and may be retrieved under accession number U26730.

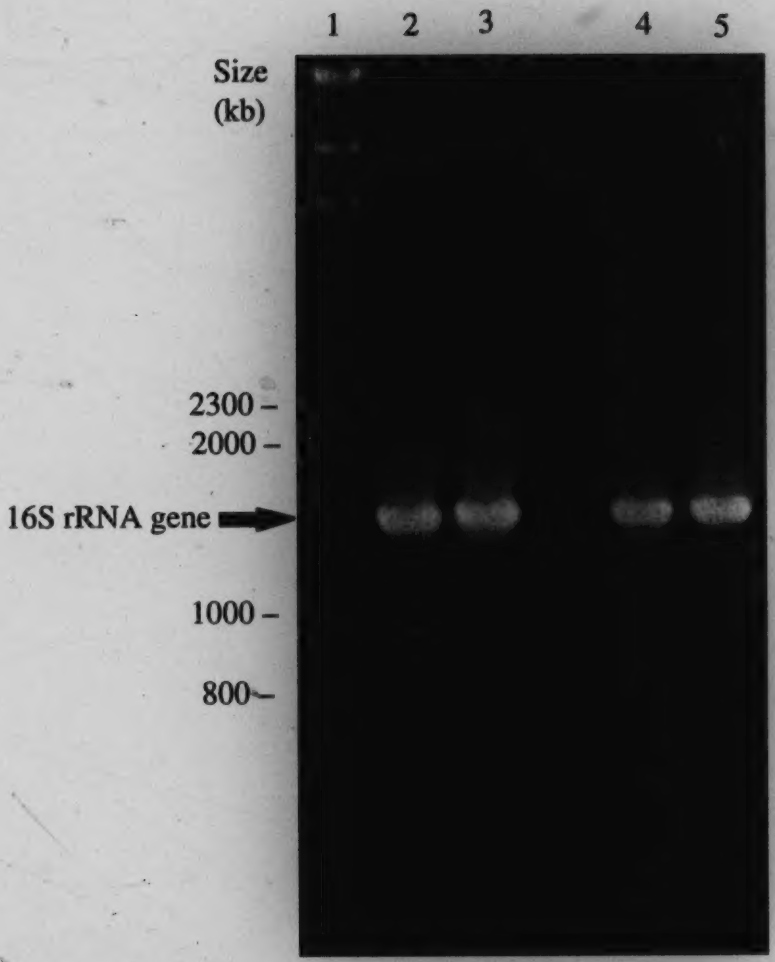
1
2
3
4
5
Size
(kb)
2300 –
2000 –
16S rRNA gene
1000 –
800 –

Figure 3-1. Amplification of *M. hyosynoviae* 16S rRNA gene by PCR. Lane 1, molecular weight marker; lanes 2-5, PCR reaction aliquots.

```
Forward     +-------->
rRNA A      <-----------+
GWS 106              +-------->
rRNA B               <----------------+
GWS 105                      +----------->
GWS 104                          <-----------+
GWS 35                               +--------->
GWS 6                                              +->
Reverse                                    <---------+
GWS 107                                    +--------->

CONSENSUS   +---------------------------------------->
            |----------|----------|----------|-------|
            0         400        800        1200    1500
```

Figure 3-2. Two-stranded sequencing strategy for *M. hyosynoviae* 16S rRNA gene. Sequencing primers used are indicated on the left of the figure.

```
   1  CCGCCCAGAGTTTGATCCTGGCTCAGGATGAACGCTGGCTGTGTGCCTAATACATGCATGTCGAGCGGGA   70

  71  GTAGCAATACTCTAGCGGCAAATGGGTGAGTAACACGTGCTTAATCTACCTTTTAGATTGGAATACCCAA  140
                                                 A
 141  TGGAAACATTGGTTAATGCCGGATAAGTATGAAATCGCATGATTTCGTTATGAAAGAAGCGTTTGCTTCA  210
      C
 211  CTAAGAGATGAGGGTGCGGAACATTAGCTAGTTGGTGAGGTAATGGCCCACCAAGGCTATGATGTTTAGC  280
                                             GWS 106
                                                                          G
 281  CGGGTCGAGAGACTGAACGGCCACATTGGGACTGAGATACGGCCCAAACTCCTACGGGAGGCAGCAGTAG  350

 351  GGAATATTCCACAATGAGCGAAAGCTTGATGGAGCGACACAGCGTGCACGATGAAGGCCCTCGGGTTGTA  420

 421  AAGTGCTGTTGCAAGGGAAGAGAAAGCAGTTGAGGAAATGCAACTGAACTGACGGTACCTTGTTAGAAAG  490
                              rRNA 1
 491  CGATGGCTAACTATGTGCCAGCAGCCGCGGTAATACATAGGTCGCAAGCGTTATCCGGAATTATTGGGCG  560

 561  TAAAGCGTTCGTAGGCTGTTTATTAAGTCTGGAGTCAAATCCCAGGGCTCAACCCTGGCTCGCTTTGGAT  630
                              GWS 105
                                                       A.
 631  ACTGGTAAACTAGAGTTGGATAGAGGTAAGCGGAATTCCATGTGAAGCGGTGAAATGCGTAGATATATGG  700

 701  AAGAACACCAAAGGCGAAGGCAGCTTACTGGGTCTATACTGACGCTGAGGGACGAAAGCGTGGGGAGCAA  770

 771  ACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGATCATTAGTCGGTGGCCAATCACTGACGCA  840
        GWS 35
                                                            rRNA 2
 841  GCTAACGCATTAAATGATCCGCCTGAGTAGTATGCTCGCAAGAGTAAAACTTAAAGGAATTGACGGGGAC  910

 911  CCGCACAAGCGGTGGAGCATGTGGTTTAATTTGAGGATACGCGGAGAACCTTACCCACTCTTGACATCCT  980

 981  TCGCAAAGCTATAGAGATATAGTGGAGGTTAACGGAGTGACAGATGGTGCATGGTTGTCGTCAGCTCGTG  1050

1051  TCGTGAGATGTTTGGTCAAGTCCTGCAACGAGCGCAACCCCTATCTTTAGTTACTAACGAGTCATGTCGA  1120
                    GWS 107

1121  GGACTCTAGAGATACTGCCTGGGTAACTGGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCTCTTAC  1190
                          GWS 104
1191  GAGTGGGGCTACACACGTGCTACAATGGTCGGTACAAAGAGAAGCAATATGGCGACATGGAGCAAATCTC  1260

1261  AAAAAGCCGATCTCAGTTCGGATTGGAGTCTGCAATTCGACTCCATGAAGTCGGAATCGCTAGTAATCGT  1330
                     rRNA 3
                                                          T
1331  AGATCAGCTTCGCTACGGTGAATACGTTCTCGGGTCTTGTACACACCGCCCGTCACACCATGGGAGCTGG  1400

1401  TAATACCCAAAGTCGGTTTGCTAACCTCGGAGGCAACTGCCTAAGGTAGGACTGGTGACTGGGGTGAAGT  1470

1471  CGTAACAAGGTATCCCTACCGGGCTAGAG  1499
```

Figure 3-3. Sequence of *M. hyosynoviae* 16S rRNA. Internal sequencing primers are indicated with an underline (positive strand) or overline (negative strand).

3.1.2 Copy Number

Digestion of mycoplasma genomic DNA (which is A+T rich) with restriction enzymes whose recognition sites are rich in A and T residues will presumably result in a large number of relatively small DNA fragments. *M. hyosynoviae* genomic DNA digested with *Alu*I, *Dra*I, *Eco*RI, *Hin*DIII, *Sau*3AI, *Ssp*I, and *Xba*I was separated on a 1% agarose gel, transferred to a nylon membrane, and probed with end-labelled oligonucleotide GWS 104 (one of the 16S-specific sequencing primers) in order to determine the number of 16S rRNA genes present in the *M. hyosynoviae* genome. Autoradiography after washing under moderate stringency (Figure 3-4) shows a single hybridizing band in each lane. Similar results were seen when oligonucleotide GWS 106 (also a sequencing primer) was used as a probe (data not shown), indicating that only one copy of the gene is present.

3.2 Phylogenetic analysis of *M. hyosynoviae* and *M. hyopharyngis*

Because the porcine mycoplasmas *M. hyorhinis*, *M. hyopneumoniae*, and *M. flocculare* had previously been shown to be closely related (Stemke et al. 1992), it was investigated whether *M. hyosynoviae* and another isolate from swine, *M. hyopharyngis*, also belong to the same clade or are more distantly related. Preliminary alignments of the 16S rRNA gene sequences of *M. hyosynoviae* and *M. hyopharyngis* (GenBank accession number U58997) with those of a variety of mollicutes indicated that *M. hyosynoviae* belongs to the *Mycoplasma hominis* group (Maniloff 1992b) while *M. hyopharyngis* belongs to the phylogenetic group containing *Mycoplasma fermentans*. The *M. hyosynoviae* and *M. hyopharyngis* sequences were subjected to maximum likelihood

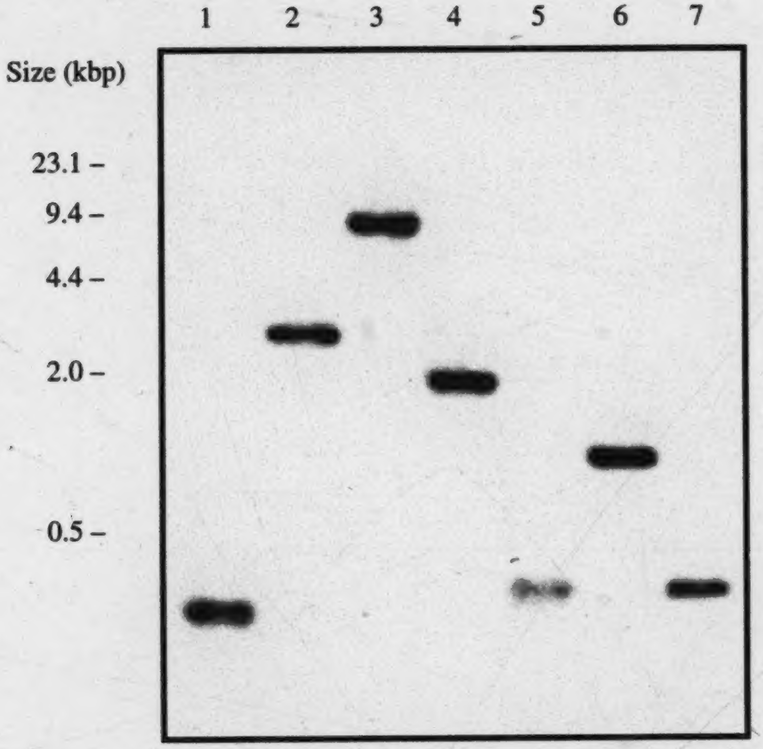
1
2
3
4
5
6
7
Size (kbp)
23.1 –
9.4 –
4.4 –
2.0 –
0.5 –

Figure 3-4. 16S rRNA gene copy number in *Mycoplasma hyosynoviae*. A Southern blot of *M. hyosynoviae* genomic DNA digested with *Alu*I, *Dra*I, *Eco*RI, *Hin*DIII, *Sau*3AI, *Ssp*I, and *Xba*I (lanes 1-7, respectively) was probed with end-labelled 16S rRNA-specific oligo GWS104.

analysis along with related organisms from their respective groups. It was determined that the closest characterized relative of *M. hyosynoviae* is *Mycoplasma orale* (97% 16S rRNA sequence identity), while that of *M. hyopharyngis* is *Mycoplasma lipophilum* (97% identity). A summary phylogenetic tree of the maximum likelihood analysis is presented in Figure 3-5.

A phylogenetic tree with the same topology was obtained when the sequence alignments were subjected to maximum parsimony analysis. Bootstrapping of the data indicated that the branching orders on the tree were statistically supported, although *M. hyosynoviae* and *M. orale* form a monophyletic group separate from *Mycoplasma salivarium* with a confidence level of only 67%, while the other branches on the tree were supported with >90% confidence.

3.3 Probes for use as genetic markers

M. hyopneumoniae genomic DNA was used as template for the PCR amplification of a number of genes which would be used to determine and compare the genetic organization of the *M. hyopneumoniae* and *M. flocculare* genomes (Table 3-1). Most of the primers (Table 2-2) used were degenerate oligonucleotides designed to amplify common housekeeping genes from a variety of bacteria. Four primer sets were designed to specifically amplify the *M. hyopneumoniae* genes for ribonucleotide reductase, lactate dehydrogenase, ciliary adhesin, and a 46 kDA antigenic lipoprotein.

Cloned mycoplasma genomic fragments were also used as probes to place genetic markers upon the maps. Three of these (the genes for 5S rRNA, glycine-tRNA, and EF-Tu) were previously characterized genes, but 16 random *M. hyopneumoniae* genomic

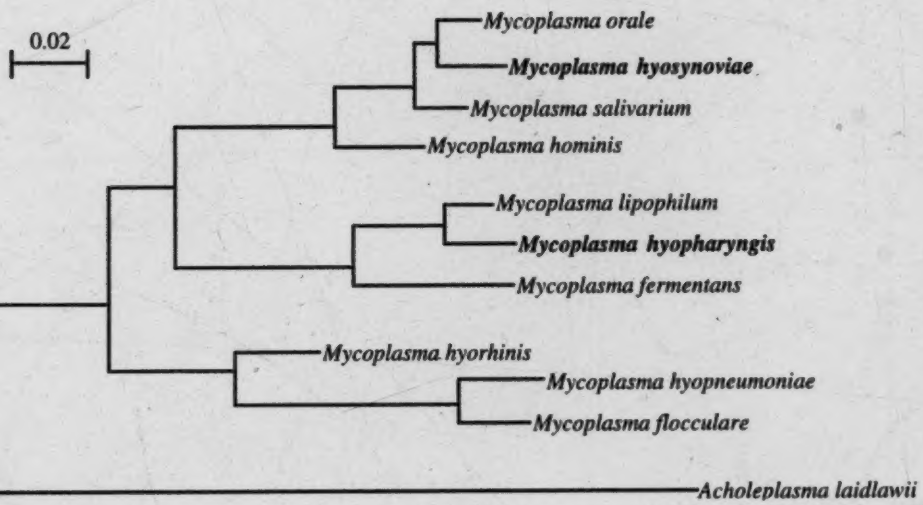
0.02
Mycoplasma orale
Mycoplasma hyosynoviae
Mycoplasma salivarium
Mycoplasma hominis
Mycoplasma lipophilum
Mycoplasma hyopharyngis
Mycoplasma fermentans
Mycoplasma hyorhinis
Mycoplasma hyopneumoniae
Mycoplasma flocculare
Acholeplasma laidlawii

Figure 3-5. Unrooted phylogenetic tree for *M. hyosynoviae* and *M. hyopharyngis*. Aligned 16S rRNA sequences from *M. hyosynoviae*, *M. hyopharyngis,* and other mollicutes were subjected to maximum likelihood analysis. Scale bar indicates expected number of nucleotide substitutions per site.

Table 3-1. PCR products used for probing digests of mycoplasma DNA. Primers used are indicated in Table 2.2.

Gene	Gene function	Size (bp)
16S rRNA	*M. hyopneumoniae* 16S ribosomal RNA	1500
adh	*M. hyopneumoniae* 94 kDa ciliary adhesin	820
dnaA	chromosomal replication initiator protein	800
gltS	glutaminyl-tRNA synthetase	800
gyrA	DNA gyrase subunit A	320
hsp60	60 kDa heat shock protein	600
ldh	lactate dehydrogenase	520
nrdF	ribonucleotide reductase R2 subunit	750
p46	*Mycoplasma hyopneumoniae* 46 kDa surface antigen	1200
pyk	pyruvate kinase	150
recA	DNA recombinase A	320
rpoB	RNA polymerase beta subunit component	400
rpoD	RNA polymerase sigma factor	100
ung	uridylyl-N-glycosylase	300
valS	valyl-tRNA synthetase	1200

fragments cloned into pBSII were also made available, along with partial sequences from both ends of the cloned insert. Ten of these clones showed sequence homology to genes found in public databases (Table 3-2). None of the remaining clones could be identified, although two of them (13-1a and B28-1b) appeared to be repetitive *M. hyopneumoniae* DNA elements, as indicated by hybridization (see below).

3.4 Mapping the M. flocculare ATCC 27716 genome

3.4.1 Restriction digests and CHEF electrophoresis

The construction of a physical map of a bacterial chromosome requires the use of restriction enzymes which cut the genomic DNA at relatively infrequent sites but often enough to produce an informative map. *M. flocculare* ATCC 27716 genomic DNA was digested with *Apa*I, *Asp*718, and *Sal*I, enzymes with recognition sites rich in guanosine and cytosine residues which had previously been shown to produce a low number of large restriction fragments from the *M. flocculare* genome (Huang and Stemke 1992). Separation of the DNA fragments by contour-clamped homogenous electric field electrophoresis (CHEF) (Figure 3-6) showed that ten bands resulted from digestion with *Apa*I, *Asp*718 digestion gave eight bands, and eight bands were visible after *Sal*I digestion. Individual bands were given a number based upon the digestion reaction which produced them (*Apa*I digestion of *M. flocculare* DNA was referred to as reaction 7, *Asp*718 as 8, and *Sal*I as 9) and their relative size compared to other bands generated by the reaction, with the letters a, b, or c appended if required to distinguish multiple fragments of the same size in a single band. Therefore, the largest band from *Apa*I digestion of *M. flocculare* was identified as band 7.1, and so forth. The sizes of the bands were determined by comparison to molecular weight standards under a variety of electrophoretic conditions

Table 3-2. Cloned DNA used for probing digests of mycoplasma DNA. Unless otherwise indicated, all clones are from *M. hyopneumoniae*.

Clone name	Gene product (gene symbol)	Size (bp)
M13 Mf5S	*M. flocculare* 5S rRNA	650
Gly-tRNA	*M. mycoides* glycine-tRNA (*glyT*)	750
EF-Tu	*M. pneumoniae* elongation factor Tu (*tuf*)	1600
5-1b	CTP synthetase* (*ctpS*) (10^{-8}; *Methanobacterium thermoautotrophicum*)	2300
B5-1b	Permease* (0.058; *Lactococcus lactis*)	160
B6-1a	Phosphotransferase system enzyme II* (*ptsII*) (0.19; *Klebsiella pneumoniae*)	330
B7-1b	N.D.†	200
B8-1b	Carbohydrate kinase* (*pfkB*) (0.12; *Archaeglobus fulgidus*)	180
9-1b	23S ribosomal RNA	560
B11-1b	N.D.	290
13-1a	*M. hyopneumoniae* repetitive element‡	1200
B14-1b	N.D.	390
B18-1b	Preprotein translocase* (*secA*) (10^{-34}; *Bacillus subtilis*)	330
19-1a	S-adenosylmethionine synthetase* (*samS*) (10^{-13}; *B. subtilis*)	490
B19-1b	Isoleucyl-tRNA synthetase* (*ileS*) (10^{-5}; *M. genitalium*)	750
B23-1a	Malonyl coenzyme A-acyl carrier protein transacylase* (*mapT*) (0.04; *Helicobacter pylori*)	2200
25-1a	Insertion sequence 1221 (IS1221)	680
26-2a	N.D.	2200
B28-1b	*M. hyopneumoniae* repetitive element‡	460

* Best match found by BLASTX searches; p-values and homolog sources are indicated.

† Not determined; no significant matches found by BLAST

‡ No significant matches found by BLAST but shown by hybridization to be present in multiple copies in the *M. hyopneumoniae* genome

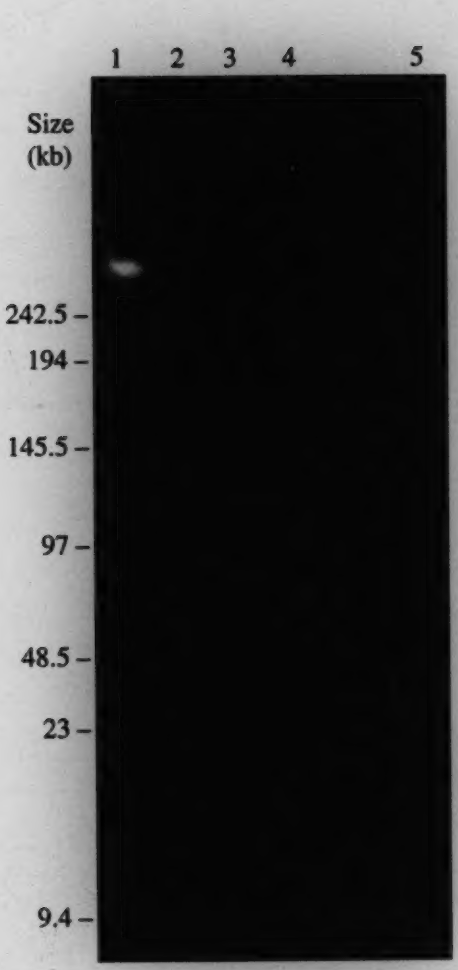
1
2
3
4
5
Size (kb)
242.5 –
194 –
145.5 –
97 –
48.5 –
23 –
9.4 –

Figure 3-6. CHEF gel electrophoresis of digested *M. flocculare* DNA. Genomic DNA was digested with *Apa*I (lane 2), *Asp*718 (lane 3), and *Sal*I (lane 4).. Molecular weight markers are lambda genome concatamers (lane 1) and *Hin*DIII digested lambda DNA (lane 5).

which optimally separated fragments of different sizes. Restriction fragment sizes from the three digest reactions are summarized in the first two columns of Tables 3-3, 3-4, and 3-5. The relative fluorescence intensity of band 9.5 indicated that it contained at least two, possibly three similarly sized restriction fragments. In order for the sum total of all fragments from each digest to give a consistent genome size for *M. flocculare*, it was assumed that band 9.5 did contain three 75 kb fragments, which was verified by the hybridization data (see below). Frequently, two partial digestion products appeared as faint bands of approximately 160 kb and 115 kb in the *SalI* digest, as previously noted (Huang and Stemke 1992). These bands did not give substantial hybridization signals and were subsequently ignored. The total size of the *M. flocculare* genome was in good agreement between all three restriction digests, approximately 900 kb.

3.4.2 Construction of the physical map

The order of the restriction fragments from each digest on the *M. flocculare* chromosome was determined by examining hybridization (and therefore overlap) between fragments from different digests. DNA was extracted from each of the individual gel bands, labelled by random priming, and used to probe Southern blots of CHEF gels of all three digests. The hybridization results from these experiments are listed in Tables 3-3, 3-4, and 3-5 and a sample autoradiograph is shown in Figure 3-7. Based on the overlapping restriction fragments, a physical map of the circular *M. flocculare* chromosome was deduced (represented linearly in Figure 3-8). The precise order of fragments 8.6 and 8.7 could not be determined because both were wholly contained within the large fragments 7.1 and 9.1.

Table 3-3. *M. flocculare Apa*I restriction fragment size and hybridization data.

*Apa*I fragment	Size (kb)	Fragments hybridized	
		*Asp*718	*Sal*I
7.1	220	8.2, 8.3, 8.6, 8.7	9.1, 9.5, 9.7
7.2	125	8.2, 8.3	9.2, 9.5
7.3	105	8.4, 8.5, 8.8	9.5, 9.6
7.4	95	(weak signal)	9.4, 9.5
7.5	85	8.3	9.1, 9.2, 9.4, 9.5
7.6	80	8.1	9.3, 9.5
7.7	70	8.1	9.3, 9.4
7.8	60	8.1	9.5, 9.8
7.9	40	8.1	9.2, 9.5, 9.8
7.10	20	8.2, 8.5	9.5
Total	900		

Table 3-4. *M. flocculare Asp*718 restriction fragment size and hybridization data.

*Asp*718 fragment	Size (kb)	Fragments hybridized	
		*Apa*I	*Sal*I
8.1	400	7.2, 7.4, 7.6, 7.7, 7.8, 7.9	9.2, 9.3, 9.4, 9.5, 9.8
8.2	155	7.1, 7.10	9.1, 9.5, 9.7
8.3	140	7.1, 7.2, 7.5	9.1, 9.2, 9.4, 9.5
8.4	60	7.3	9.5, 9.6
8.5	55	7.3, 7.10	9.5, 9.6
8.6	45	7.1	9.1
8.7	30	7.1	9.1
8.8	15	7.3	9.6
Total	900		

Table 3-5. *M. flocculare Sal*I restriction fragment size and hybridization data.

*Sal*I fragment	Size (kb)	Fragments hybridized *Apa*I	Fragments hybridized *Asp*718
9.1	220	7.1, 7.5	8.2, 8.3, 8.6, 8.7
9.2	165	7.2, 7.5, 7.9	8.1, 8.3
9.3	90	7.6, 7.7	8.1
9.4	85	7.4, 7.7	8.1
9.5*	75	7.1, 7.3, 7.4, 7.5, 7.6, 7.8, 7.10	8.1, 8.2, 8.3, 8.4,8.5
9.6	60	7.3	8.4, 8.5, 8.8
9.7	30	7.1	8.2
9.8	30	7.8, 7.9	8.1
Total	905		

* Triplet band

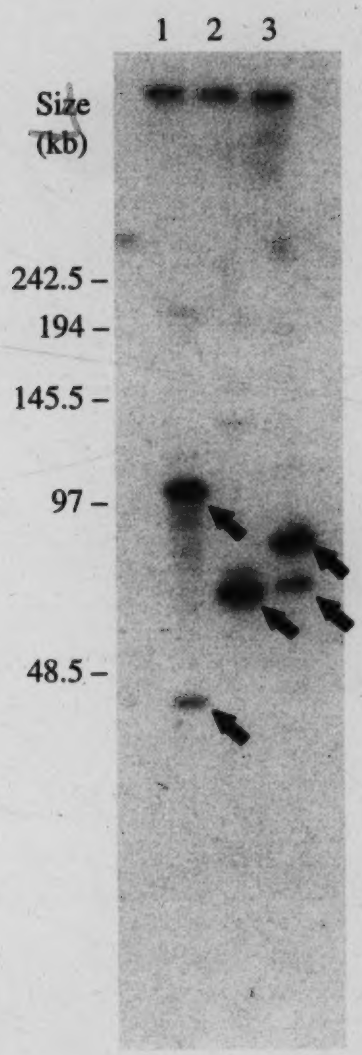
1
2
3
Size (kb)
242.5 –
194 –
145.5 –
97 –
48.5 –

Figure 3-7. Southern blot of CHEF separated *M. flocculare* DNA digests probed with random-primer labelled fragment 8.5. Arrows indicate overlapping fragments: lane 1, *Apa*I fragments 7.3 and 7.10; lane 2, *Asp*718 fragment 8.5; lane 3, *Sal*I fragments 9.5 and 9.6.

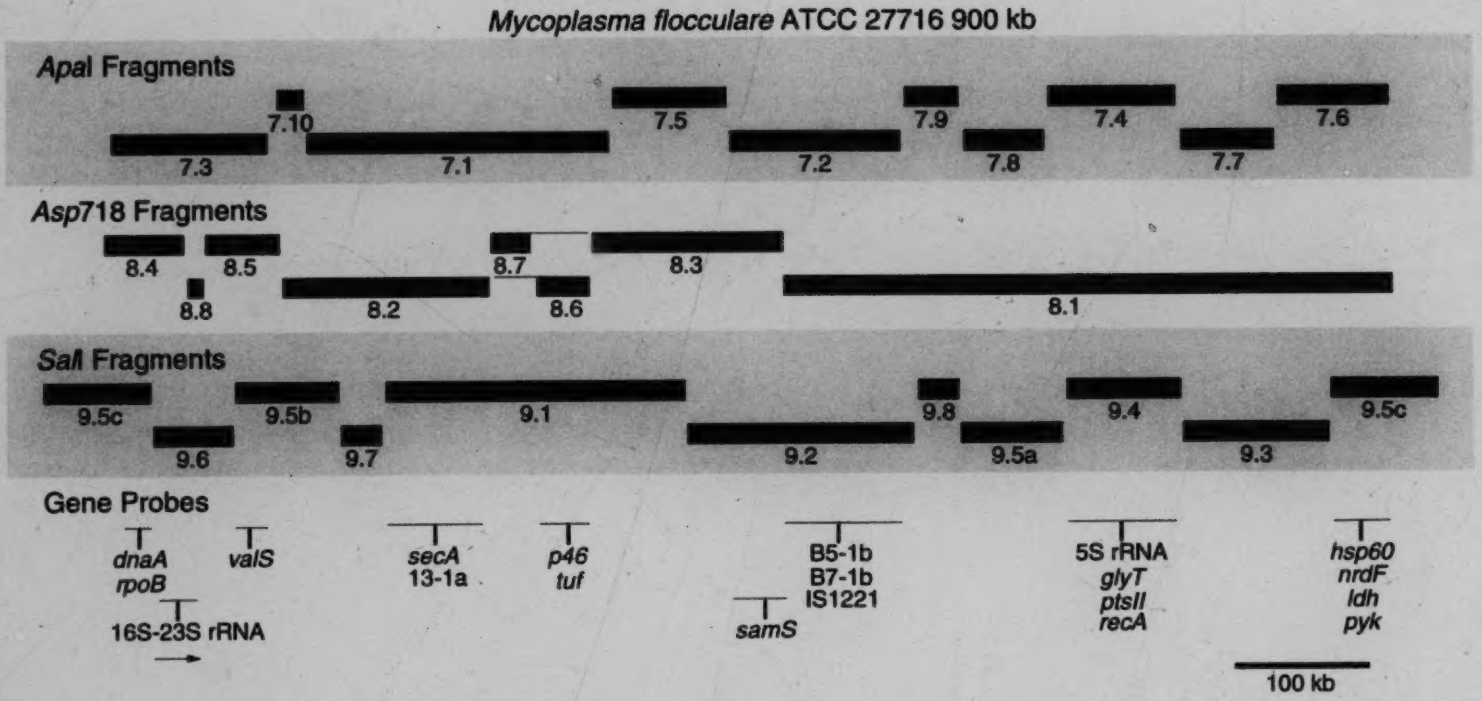
Mycoplasma flocculare ATCC 27716 900 kb
*Apa*I Fragments
7.3
7.10
7.1
7.5
7.2
7.9
7.8
7.4
7.7
7.6
*Asp*718 Fragments
8.4
8.8
8.5
8.2
8.7
8.6
8.3
8.1
*Sal*I Fragments
9.5c
9.6
9.5b
9.7
9.1
9.2
9.8
9.5a
9.4
9.3
9.5c
Gene Probes
dnaA
rpoB
16S-23S rRNA
valS
secA
13-1a
p46
tuf
samS
B5-1b
B7-1b
IS1221
5S rRNA
glyT
ptsII
recA
hsp60
nrdF
ldh
pyk
100 kb

Figure 3-8. Physical and genetic map of *M. flocculare* genome, as determined by CHEF electrophoresis of macrorestriction fragments and Southern hybridization. Individual restriction fragments are represented by rectangles, horizontal lines are ambiguity bars covering the area within which each element is located. Multiple elements under one ambiguity bar are not listed in any particular order. Refer to Tables 3-1 and 3-2 for identification of gene probes. Where possible, markers are labelled with their respective gene symbols. Arrow indicates direction of transcription of rRNA operon.

3.4.3 Placement of genetic markers on the map

The PCR products and cloned mycoplasma genomic fragments listed in Tables 3-1 and 3-2 were used to probe Southern blots of CHEF separated *M. flocculare* genome digests. Because most of the probes were from other species of mycoplasmas, hybridizations and washes were performed at low stringency to allow the heterologous probes to form duplexes with *M. flocculare* DNA. In total, 21 of these genetic markers successfully hybridized to *M. flocculare* macrorestriction fragments (Table 3-6) and were placed on the physical map (Figure 3-8). The origin of chromosomal replication, as indicated by the *dnaA* gene, was located to the region overlapped by fragments 7.3, 8.4, and 9.5c. Although most probes hybridized unambiguously to one fragment from each digest, the probes for the EF-Tu and P46 genes showed nonspecific hybridization to each fragment of the *M. flocculare* genome. The intensity of the hybridization signal from fragment 8.6 relative to surrounding fragments (in both cases), however, allowed localization of the genes to that region.

3.5 Mapping the *M. hyopneumoniae* strain J genome

3.5.1 Restriction digests and CHEF electrophoresis

Intact *M. hyopneumoniae* strain J genomic DNA was digested with a variety of restriction enzymes which have recognition sites rich in G and C residues, and was compared with *Apa*I, *Apa*LI, and *Asp*718 digests of *M. hyopneumoniae* ATCC 25095 and *Apa*I, *Apa*LI, and *Sal*I digests of *M. flocculare* ATCC 27716 (Figure 3-9). A number of common restriction fragments were found in the two *M. hyopneumoniae* strains, but no common fragments could be recognized between *M. flocculare* and *M. hyopneumoniae*.

Table 3-6. Hybridization of genetic probes with *M. flocculare* macrorestriction fragments. See tables 3-1 and 3-2 for identification of probes.

	Fragments hybridized		
Probe	*Apa*I	*Asp*718	*Sal*I
23S rRNA	7.3	8.8	9.6
16S rRNA	7.3	8.4	9.6
5S rRNA	7.4	8.1	9.4
dnaA	7.3	8.4	9.5
glyT	7.4	8.1	9.4
hsp60	7.6	8.1	9.5
IS1221	7.2	8.1	9.2
ldh	7.6	8.1	9.5
nrdF	7.6	8.1	9.5
p46	N.D.*	8.6	N.D.
ptsII	7.4	8.1	9.4
pyk	7.6	8.1	9.5
recA	7.4	8.1	9.4
rpoB	7.3	8.4	9.5
samS	7.2	8.3	9.2
secA	7.1	8.2	9.1
tuf	N.D.	8.6	N.D.
valS	7.3	8.5	9.5
B5-1b	7.2	8.1	9.2
B7-1b	7.2	8.1	9.2
13-1a	7.1	8.2	9.1

* N.D., not determined due to high levels of nonspecific hybridization

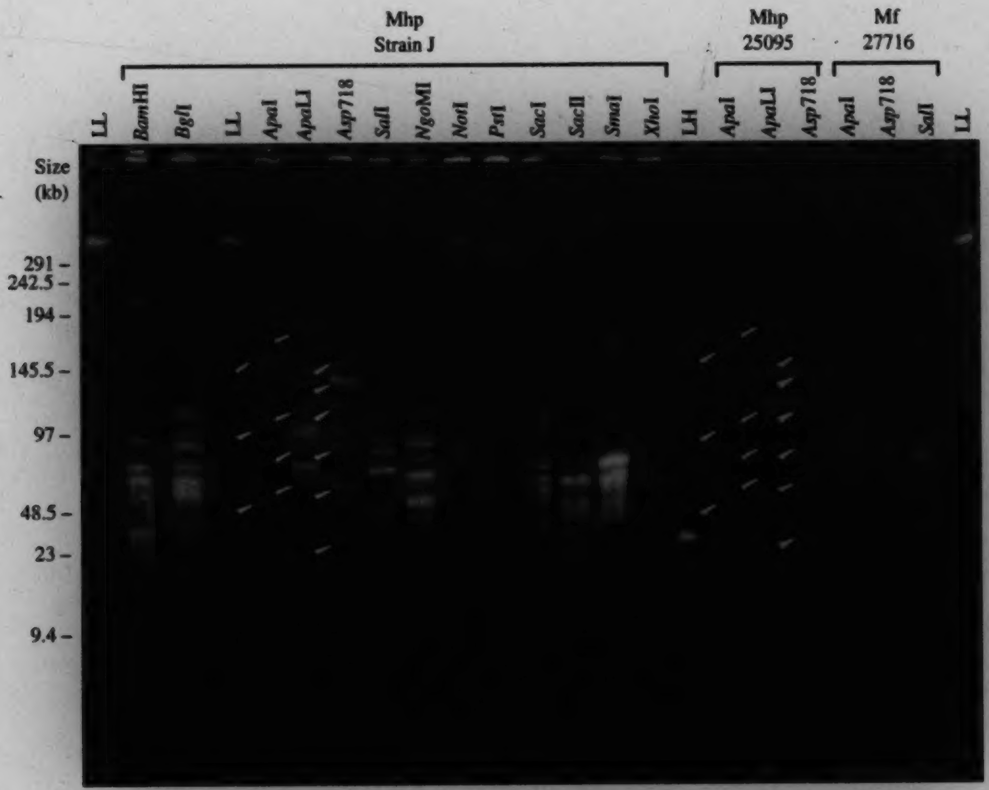
Mhp
Strain J
Mhp
25095
Mf
27716
LL
BamHI
BglI
LL
ApaI
ApaLI
Asp718
SalI
NgoMI
NotI
PstI
SacI
SacII
SmaI
XhoI
LH
ApaI
ApaLI
Asp718
ApaI
Asp718
SalI
LL
Size
(kb)
291 -
242.5 -
194 -
145.5 -
97 -
48.5 -
23 -
9.4 -

Figure 3-9. CHEF of porcine mycoplasma genomic DNA digested by restriction enzymes with GC-rich recognition sites. Genomic DNA source and enzymes used to digest are indicated above lanes. Abbreviations: Mhp, *M. hyopneumoniae*; Mf, *M. flocculare*; LL, lambda concatomer ladder; LH, lambda *Hin*DIII digest. Arrowheads indicate bands shared between *M. hyopneumoniae* strains J and ATCC 25095 when digested with the same enzyme.

*Apa*I, *Apa*LI, *Asp*718, and *Sal*I were chosen for physical mapping of the *M. hyopneumoniae* strain J genome on the basis of a relatively low number of high molecular weight restriction fragments generated (Figure 3-10). Fragments were named according to the same convention used for *M. flocculare* digest fragments. *Apa*I digestion of *M. hyopneumoniae* chromosomes (reaction 1) yielded 13 identifiable bands, *Apa*LI (reaction 2) and *Asp*718 (reaction 3) digests both gave 10 bands, and *Sal*I digestion (reaction 10) gave 11 bands. The sizes of the bands were determined by comparison to molecular weight standards under a variety of electrophoretic conditions which optimally separated fragments of different sizes. Restriction fragment sizes from the four digest reactions are summarized in the first two columns of Tables 3-7, 3-8, 3-9, and 3-10. Relative band fluorescence intensities and hybridization data indicated that a number of bands contained multiple fragments of similar size, as indicated in the Tables. Taking this into account, the size of the *M. hyopneumoniae* genome as determined by averaging the totals from each of the restriction digests is approximately 1080 kb.

3.5.2 Restriction fragment hybridization

M. hyopneumoniae DNA extracted from individual bands from CHEF gels was used to probe Southern blots of CHEF separated restriction fragments from all four *M. hyopneumoniae* genomic digests in order to identify overlapping fragments. The hybridization results from these experiments is summarized in Tables 3-7, 3-8, 3-9, and 3-10. Based upon this data, most of the restriction fragments could be ordered into three groups of overlapping fragments. Inconsistencies due to the number of similarly sized fragments present in a single digest, contamination of probes with neighboring DNA fragments, and the inablility to detect many of the smaller fragments by hybridization

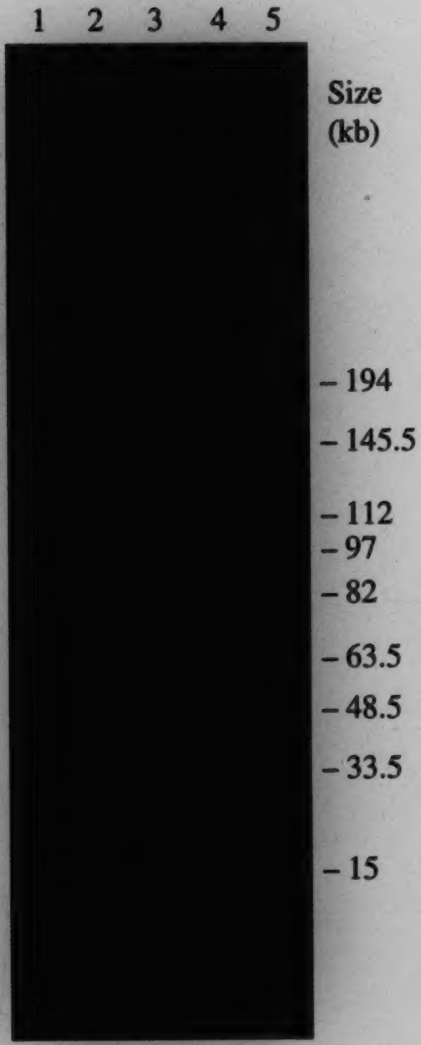
1
2
3
4
5
Size
(kb)
– 194
– 145.5
– 112
– 97
– 82
– 63.5
– 48.5
– 33.5
– 15

Figure 3-10. CHEF gel electrophoresis of digested *M. hyopneumoniae* strain J DNA. Genomic DNA was digested with *Apa*I (lane 1), *Apa*LI (lane 2), *Asp*718 (lane 3), and *Sal*I (lane 4). Molecular weight marker (lane 5) is PFGE Midrange Marker I (NEB).

Table 3-7. *M. hyopneumoniae Apa*I restriction fragment size and hybridization data.

*Apa*I fragment	Approximate Size (kb)	Fragments hybridized		
		*Apa*LI	*Asp*718	*Sal*I
1.1	285	2.4, 2.5, 2.9, 2.11	3.1, 3.3, 3.6	10.4, 10.5, 10.6, 10.7, 10.8
1.2	145	2.2, 2.7	3.2, 3.5	10.3, 10.6
1.3	140	2.1, 2.2	3.2	10.2, 10.3, 10.6
1.4	105	2.3, 2.8	3.2, 3.6	10.1, 10.6
1.5	100	2.5, 2.7	3.2, 3.4	10.5, 10.6
1.6	60	2.6	3.2	10.5, 10.6
1.7*	45	2.3, 2.6, 2.7	3.2, 3.3, 3.5	10.1, 10.6
1.8	45	2.8, 2.9, 2.10	3.2, 3.6	10.6, 10.7
1.9*	25	2.3, 2.7	3.5	10.6
1.10	20	2.6	3.2, 3.3	10.6
1.11	15	2.2, 2.3	3.7	10.1, 10.6
1.12	15	N. D.†	N. D.	N. D.
1.13	10	N. D.	N. D.	N. D.
Total	1080			

* Doublet bands.
† Not determined.

Table 3-8. *M. hyopneumoniae ApaLI* restriction fragment size and hybridization data.

*Apa*LI fragment	Approximate Size (kb)	Fragments hybridized		
		*Apa*I	*Asp*718	*Sal*I
2.1	180	1.3, 1.5	3.2, 3.4	10.2, 10.5
2.2	130	1.2	3.2	10.3, 10.6, 10.10
2.3	110	1.4, 1.7, 1.9	3.2, 3.5, 3.6	10.1, 10.6
2.4	95	1.1	3.1, 3.2	10.4, 10.5, 10.6
2.5*	90	1.1	3.1, 3.2, 3.3, 3.4	10.4, 10.5, 10.6, 10.8
2.6	85	1.1, 1.6, 1.7	3.2, 3.7	10.1, 10.6
2.7*	70	1.2, 1.7	3.1, 3.2, 3.5	10.6, 10.11
2.8	60	N. D.†	N. D.	N. D.
2.9*	30	1.1	3.3	10.6
2.10	10	N. D.	N. D.	N. D.
Total	1050			

* Doublet bands.
† Not determined.

Table 3-9. *M. hyopneumoniae Asp*718 restriction fragment size and hybridization data.

*Asp*718 fragment	Approximate Size (kb)	Fragments hybridized		
		*Apa*I	*Apa*LI	*Sal*I
3.1	140	1.1	2.4, 2.5	10.4, 10.5
3.2*	125	1.2, 1.3, 1.4, 1.5, 1.6, 1.8	2.1, 2.2, 2.6, 2.8	10.1, 10.2, 10.3, 10.6, 10.10
3.3	110	1.1, 1.8, 1.11	2.5, 2.7, 2.9	10.7, 10.8
3.4	95	1.5	2.5, 2.7, 2.9	10.7, 10.8
3.5	80	1.2, 1.6	2.3, 2.7	10.6
3.6†	50	1.1, 1.4	2.3, 2.5	10.1, 10.4, 10.6, 10.8
3.7	45	1.7, 1.12	2.6	10.1, 10.6
3.8†	30	1.5	2.1	10.5
3.9†	20	1.2	2.7	10.6
3.10	10	1.9	2.3	10.6
Total	1095			

* Triplet band.
† Doublet bands.

Table 3-10. *M. hyopneumoniae Sal*I restriction fragment size and hybridization data.

*Sal*I fragment	Approximate Size (kb)	Fragments hybridized		
		*Apa*I	*Apa*LI	*Asp*718
10.1	300	1.4, 1.6, 1.7, 1.8	2.3, 2.5, 2.7	3.2, 3.6, 3.7
10.2	110	1.3	2.1	3.2
10.3	90	1.2, 1.3	2.2	3.2
10.4	85	1.1	2.4, 2.5	31, 3.2, 3.6
10.5*	75	1.5	2.4, 2.5	3.1, 3.2, 3.4
10.6†	65	1.2, 1.5, 1.8, 1.10	2.2, 2.3, 2.5, 2.7	3.2, 3.5, 3.8
10.7‡	40	1.1	2.5, 2.7, 2.9	3.3, 3.6
10.8‡	37	1.1	2.5, 2.7, 2.9	3.3, 3.6
10.9	33	1.1	2.5	3.2, 3.3
10.10	30	1.2	2.2	3.2
10.11	20	1.2	2.7	3.5
Total	1090			

* Doublet band.
† Triplet band.
‡ 10.7 and 10.8 probes were copurified.

prevented the construction of a complete physical map of the *M. hyopneumoniae* genome at this point.

3.5.3 Cosmid libraries

High molecular weight genomic DNA was extracted from cultures of *M. flocculare* 27716 and *M. hyopneumoniae* strain J. The isolated DNA was subjected to differential PCR (Stemke et al. 1994b) using (in a single reaction) *M. flocculare* 16S rRNA gene-specific primer GWS 118, *M. hyopneumoniae*-specific primer GWS 119, and common primer GWS 120. The presence of a single 238 bp product in the *M. hyopneumoniae* reactions and a 400 bp product in the *M. flocculare* reaction (Figure 3-11) confirmed the identity of the cultures and verified that no cross-contamination of the cultures occurred. Genomic DNA from each culture was partially digested with *Eco*RI, size fractionated by sucrose gradient centrifugation, and the fractions containing DNA fragments 25-40 kb in length were pooled to construct the libraries. DNA from the pooled fractions was treated with alkaline phosphatase, ligated into prepared cosmid vector pcosRW2 (Wenzel and Herrmann 1988), packaged in phage lambda packaging extracts, and transfected into *E. coli*, which was plated on selective media. From the *M. flocculare* cosmid library, 672 clones were transferred to microtitre plates for growth and storage as stock cultures to await future analysis.

The cross-screening method of Locke et al. (1996) was used to detect overlaps between cosmids from the *M. hyopneumoniae* genomic library and thereby to order the cosmids into contiguous segments of the genome. A manifold apparatus was used to simultaneously hybridize up to 45 probes (labelled RNA transcripts from the ends of cosmid inserts, generated from the T7 and SP6 promoter sequences present on the vector) with up to 45 target cosmids immobilized in stripes across nylon membranes. When

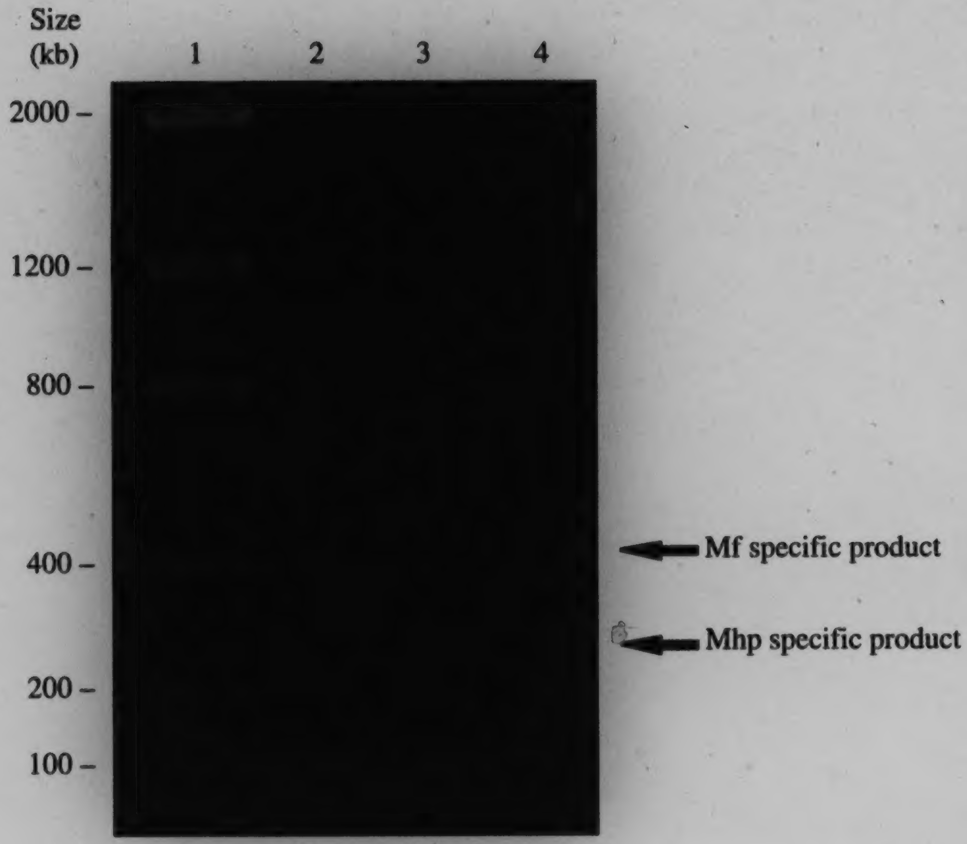
Size
(kb)
1
2
3
4
2000 –
1200 –
800 –
400 –
200 –
100 –
Mf specific product
Mhp specific product

Figure 3-11. Differential PCR of Mhp (lanes 2 and 3) and Mf (lane 4) genomic DNA, separated on 1.3% agarose gel. Lane 1, BRL molecular mass ladder.

developed (either by colorimetric or chemiluminescent detection of the DIG-labelled probes) the membrane resembled an array of small squares (Figure 3-12), in which each square marked the intersection of a hybridizing probe and target. Probe quality was monitored by observation of hybridization of a transcript with its own template and with *M. hyopneumoniae* genomic DNA also immobilized in a separate lane on the membrane. A probe which hybridized with each clone in a cross-screen was assumed to be a transcription of vector sequence (as a result of recombination in the cosmid) and was removed from further analysis. Two cosmids were considered to overlap if a transcript from one cosmid hybridized with a second cosmid, and a transcript from the second cosmid reciprocally hybridized with the first cosmid.

The strategy employed to assemble contigs was as follows. Cosmid DNA was extracted from two hundred clones picked at random from the *M. hyopneumoniae* library. These cosmids were divided into groups of approximately forty, excluding those which did not contain inserts as determined by *Eco*RI digest patterns (21 clones were excluded on this basis). Each group was cross screened with its own T7 and SP6 transcripts to identify overlapping cosmids within that group. This initial round of cross-screening resulted in 28 contigs, ranging in length from one "orphan" cosmid to seven overlapping clones. Further cross-screens between groups and comparisons of *Eco*RI restriction digest patterns of the cosmid inserts were performed to eliminate redundant contigs and establish a minimum set of overlapping cosmids. The contig flanked by cosmids 196 and 187 had a particularly high concentration of overlapping cosmids, and *Eco*RI restriction analysis of these clones (Figure 3-13) permitted the construction of a high-resolution partial *Eco*RI restriction map of this region (Figure 3-14).

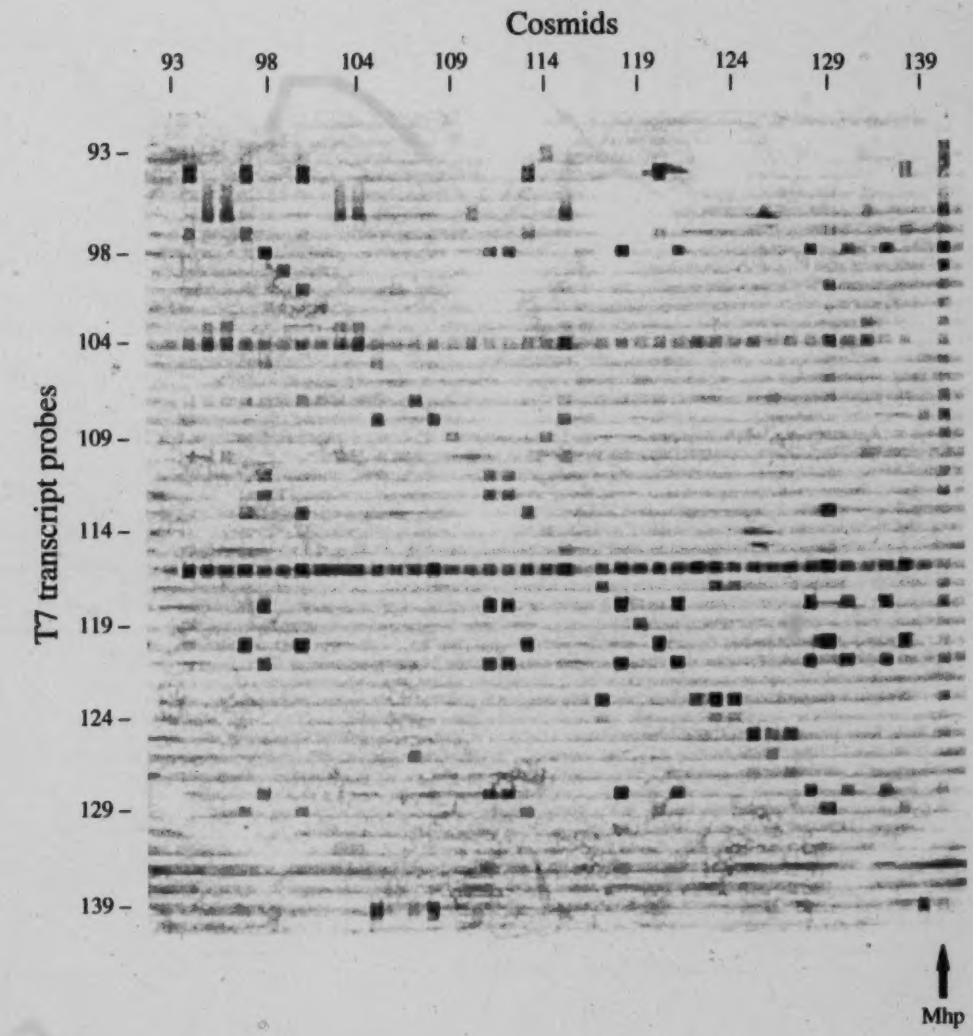
Cosmids
93
98
104
109
114
119
124
129
139
T7 transcript probes
93 -
98 -
104 -
109 -
114 -
119 -
124 -
129 -
139 -
Mhp

Figure 3-12. Cross-screen of *M. hyopneumoniae* cosmid group 93-139. Cosmid clones (vertical columns) were probed with their own T7 transcript probes (horizontal rows). The last column, marked Mhp, is *M. hyopneumoniae* genomic DNA. Clones 100 and 132-136 were excluded from this analysis.

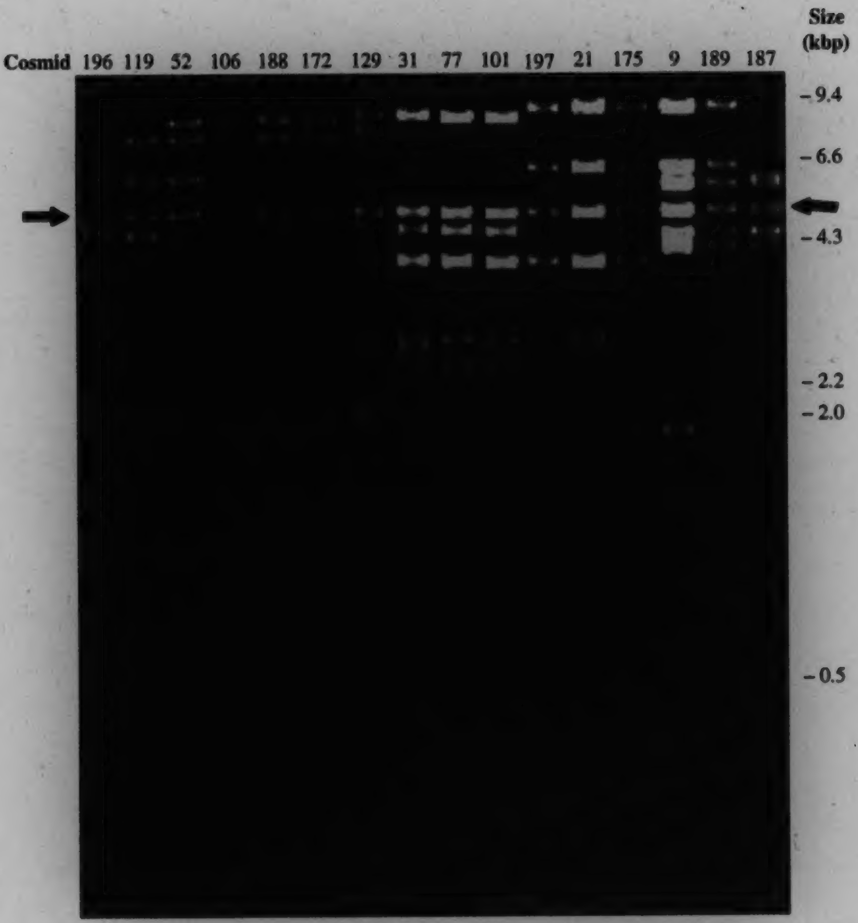
Cosmid 196 119 52 106 188 172 129 31 77 101 197 21 175 9 189 187
Size (kbp)
9.4
6.6
4.3
2.2
2.0
0.5

Figure 3-13. *Eco*RI restriction digests of overlapping clones in contig 196-187. Fragments were separated on a 0.7% agarose gel. Arrows indicate vector pcosRW2 band.

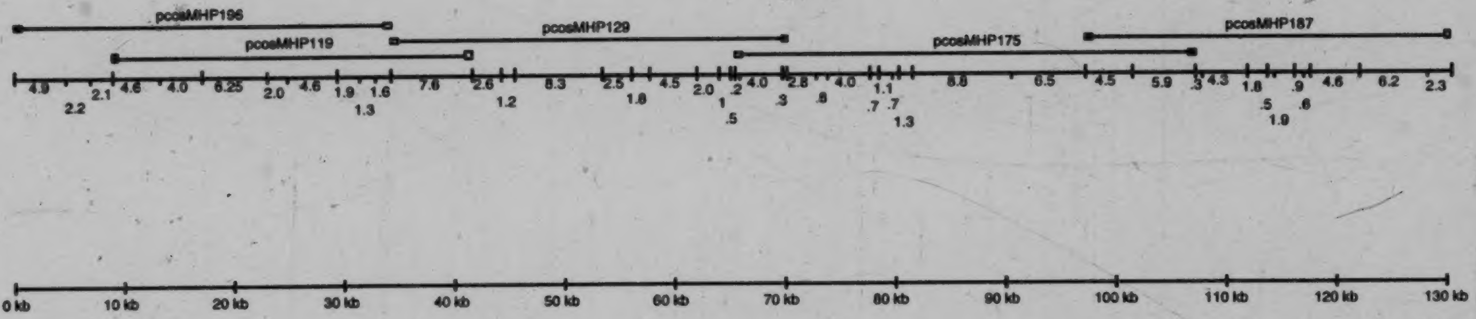
pcosMHP196
pcosMHP119
pcosMHP129
pcosMHP175
pcosMHP187
4.9
2.2
2.1
4.6
4.0
6.25
2.0
4.6
1.9
1.3
1.6
7.6
2.6
1.2
8.3
2.5
1.8
4.5
2.0
1
.5
.2
4.0
2.8
.3
.8
4.0
1.1
.7
.7
1.3
8.8
6.5
4.5
5.9
.3
4.3
1.8
.5
1.9
.9
.6
4.6
6.2
2.3
0 kb
10 kb
20 kb
30 kb
40 kb
50 kb
60 kb
70 kb
80 kb
90 kb
100 kb
110 kb
120 kb
130 kb

Figure 3-14. Partial *Eco*RI map of *M. hyopneumoniae* cosmid contig 196-187, as deduced from Figure 3-11. Fragment sizes are indicated in kb. Marks extending above and below the horizontal line indicate definitively placed *Eco*RI sites, marks below the line indicate that the precise order of the fragments separated by that restriction site is not known. Cosmids required to cover this region in a minimal set are also shown, and T7 (filled squares) and SP6 (open squares) ends are indicated.

From the analysis of the first 200 cosmid clones four contigs, consisting of two to seven overlapping cosmids, and 6 orphan cosmids were obtained (Table 3-11). The total coverage of the *M. hyopneumoniae* genome at this point was estimated at approximately 500 kb. Probes transcribed from the ends of the contigs and from the orphans were cross-screened against another batch of 350 randomly picked cosmid clones. Positive clones were transcribed and used in successive rounds of cross-screening. Through this process the contigs were enlarged and some of the contigs and orphans were merged by newly found clones (Table 3-11).

In addition to cross-screening and *Eco*RI digest pattern comparisons, cosmid placement upon the *M. hyopneumoniae* CHEF physical map (which was constructed with the assistance of cosmid probe hybridizations; see Figure 3-15 below) was also used to determine whether a cosmid was suitable for inclusion in a contig. T7 and SP6 transcripts from the cosmids included in contigs and those identified by cross-screening were used as probes against Southern blots of *M. hyopneumoniae* DNA digested by *Apa*I, *Apa*LI, *Asp*718, and *Sal*I and separated by CHEF (Table 3-12). This hybridization analysis permitted the identification of "chimeric" cosmids, whose T7 and SP6 ends hybridized to widely separated regions of the genome. Cosmids 167 and 181 were excluded from further analysis on this basis. Cosmid 277, identified by probe T7-182 during cross-screening, proved to be located distantly from 182, but was located in a region of the genome which had not yet been covered by cosmids. Subsequent cross-screening with 277 found an overlap with cosmid 229. Cosmid 57, which overlapped with 45 and had cross-screened with cosmids 259 and 274, could not be reisolated after the initial aliquot of cosmid DNA was exhausted. Later isolates from the same culture stock hybridized to the region of the genome covered by cosmids 43 and 543, suggesting that contamination with

Table 3-11. *M. hyopneumoniae* cosmid contigs and orphans identified by cross-screening.

First batch (cosmids 1-200)	Second batch (cosmids 201-350)
118-146-136-125-147-42	(57)*-259-274-276-544-46-42-147
167-187-175-129-119-196	449-509-182-214-1-45-(57)
1-45-57	395-187-175-129-119-196
102-135	543-43-450-118-146
43	277-229
107	
153	
170	
181	
182	

* Parentheses indicate that the original cosmid 57 which cross-screened with cosmids 45 and 259 was later lost.

another clone had occurred.

In an attempt to bridge the remaining gaps between contigs, transcripts from the ends of the contigs were used to probe colony blots of approximately 800 more clones picked from the cosmid library. Nearly all of the clones hybridized by this procedure either did not extend the contigs, were chimeric, or had undergone extensive deletions. Only two cosmids identified in the colony blots, designated 22.1 and 32.1, increased the length of the contigs, and the short length of cosmid insert 22.1 indicates it may have undergone a deletion.

The cosmids from each of the final five contigs were digested by *Eco*RI and the sizes of their constituent fragments were measured by gel electrophoresis in order to determine the extent of overlap between cosmids and total size of the contigs (Table 3-12). The total length of all unique *Eco*RI fragments cloned into the overlapping cosmids was calculated as 728 kb. Assuming a total genome size of 1080 kb (as determined by CHEF), this represents 67% coverage of the *M. hyopneumoniae* genome.

3.5.4 Assembly of the physical map

The probes generated by transcription from the T7 and SP6 ends of the cosmid inserts were used to complete the ordering of the *M. hyopneumoniae* restriction fragments generated by digestion with *Apa*I, *Apa*LI, *Asp*718, and *Sal*I. Probes from all of the cosmids contained in the contigs, and numerous candidates for extension of the contigs, were hybridized to Southern blots of CHEF separated DNA (Table 3-13). Overlapping fragments, as determined by hybridization with transcript probes and labelled fragments (Tables 3-7 to 3-10), were assembled into a physical map of the *M. hyopneumoniae* genome (Figure 3-15). Occasionally probes were transcribed from a region containing one

Table 3-12. Contig summary of overlapping *M. hyopneumoniae* cosmid clones. *Eco*RI fragments unique to each cosmid insert are underlined. Total coverage of the *M. hyopneumoniae* genome (size of all contigs) is 728 kb.

Contig I

259	274	276	544	46	42	147	22.1	Cosmid
7000	7000	12000	5200	5200	5900	9400	4500	
7000	7000	6800	4200	4200	3600	6400	3600	
5700	6800	5700	3200	3600	3600	3600	3600	
4600	4600	4050	3200	3150	3200	3450	3150	
3250	2200	2700	3150	3150	3200	3300	1150	*Eco*RI fragment sizes (bp)
2200	1750	2400	2700	3100	3100	3150	800	
1750	1550	2300	2300	3000	1650	1650	450	
1550	1350	2300	2300	2200	1650	1500	200	
1350	1000	2200	2200	1800	1500	1250		
1000	500	1800	1250	1650	1300	750		
500		1550	1100	1100	1250			
		1350	900	950	950			
			650	800	800			
				500				
35900	33750	45150	32350	34400	31700	34450	17450	Insert size
							187000	Total contig size

Contig II

395	187	175	129	119	196
8500	6200	8800	8300	7600	6300
6200	5900	6500	7600	6300	4900
4600	4600	5900	4500	4600	4600
4400	4500	4500	4000	4600	4600
4300	4300	4000	2600	4000	4000
4250	2300	4000	2500	2000	2200
2200	1900	2800	2000	1900	2100
	1800	1300	1800	1600	2000
	900	1100	1200	1300	1900
	600	800	1000		1600
	500	700	500		1300
	300	700	300		
		300	200		
		200			
34450	33800	41600	36500	33900	35500
					157750

Table 3-12 Continued.

Contig III

32.1	543	43	450	118	68
9100	8600	9500	9500	9600	11500
4500	5400	8600	7200	7200	5700
2700	3250	6000	3900	5700	5700
2600	2700	3900	3450	4800	4800
2300	2600	3300	3300	3450	3550
2150	2300	2350	1650	3200	3200
1900	2150	1650	1400	3200	3200
1600	2150	1350	1400	1650	1800
1400	1600	1250	1050	1500	1550
1350	1350	1100			1300
1200	1200				
1150	1000				
1100	750				
1000	500				
750					
500					
400					
300					
200					
36200	35550	39000	32850	40300	42300
					155550

Contig IV

449	509	182	214	1	45
4800	6000	17500	8400	11500	11500
4700	6000	8400	4400	6800	6800
4500	4800	4300	4300	5200	5200
4200	4800	4300	3800	2450	4500
4050	4300	2700	2700	1900	2450
3800	4300	1850	2150	1900	1900
3700	4200	900	1900	1350	1650
3450	3700		1850	1350	1350
2900	2900		1050	900	850
1150	2000		900	450	
900	2000		900	300	
	400		500	300	
38150	45400	39950	32850	34400	36200
					156000

Contig V

277	229
8100	8800
5200	4400
5100	4200
4600	4200
4500	4000
3100	2300
2300	2100
1950	1850
1900	1100
1200	950
1000	900
500	
39450	34800
	71950

Table 3-13. Hybridization of cosmid clone T7 and SP6 transcript probes to CHEF separated *M. hyopneumoniae* macrorestriction fragments

Cosmid clone	Fragments hybridized	
	T7	SP6
1	1.4, 2.3, 3.6, 10.1	1.4, 1.9, 2.3, 3.5, 3.9, 10.1
22.1	1.3, 2.1, 3.2, 10.2	1.3, 2.1, 3.2, 10.3
32.1	1.3, 2.1, 3.2, 10.2	1.5, 2.1, 3.2, 10.5
42	1.2, 2.2, 3.2, 10.3	1.2, 1.3, 2.2, 3.2, 10.3
43	1.5, 2.1, 3.4, 3.8, 10.5	1.5, 2.1, 3.2, 10.5
45	1.7, 1.9, 2.3, 3.5, 10.1, 10.6	1.4, 2.3, 3.6, 10.1
46	1.2, 2.2, 3.2, 10.3	1.2, 2.2, 3.2, 10.3
(57)*	1.5, 2.1, 3.8, 10.5	1.3, 1.5, 2.1, 3.2, 10.2, 10.5
68	1.5, 2.5, 3.4, 10.5, 10.6	1.1, 1.5, 2.5, 3.4, 10.6
118	1.5, 2.1, 2.5, 3.4, 10.5, 10.6	1.1, 2.5, 3.4, 10.6
119	1.6, 2.6, 3.2, 10.1	1.7, 2.6, 3.7, 10.1
129	1.10, 2.7, 3.3, 10.1	1.1, 1.4, 1.6, 1.7, 1.8, 1.12, 2.4, 2.6, 2.8, 2.9, 2.10, 3.1, 3.2, 3.3, 3.7, 10.1, 10.5, 10.6, 10.9
146	1.1, 2.5, 3.4, 10.6	1.1, 1.5, 2.5, 3.4, 10.6
147	1.2, 1.3, 2.2, 3.2, 10.3	1.3, 2.1, 2.2, 3.2, 10.3
153†	1.1, 2.5, 3.6, 10.4	1.8, 1.4, 2.8, 3.2, 10.1
167†	1.1, 2.5, 2.9, 2.10, 3.3, 3.6, 10.8, 10.9	1.2, 2.2, 3.2, 10.6
175	1.1, 2.7, 2.9, 3.3, 10.7	1.10, 2.7, 3.3, 10.1, 10.7
181†	1.1, 2.7, 2.9, 3.3, 10.7	1.4, 2.3, 3.6, 10.1
182	1.1, 1.4, 1.6, 1.8, 2.3, 2.4, 2.6, 2.8, 2.9, 2.10, 3.1, 3.2, 3.3, 3.6, 3.7, 10.1, 10.5, 10.9	1.4, 2.3, 2.8, 3.6, 10.1
187	1.1, 2.9, 3.3, 10.7	1.1, 2.5, 2.9, 3.3, 10.9, 10.7
196	1.6, 2.6, 3.2, 10.1	1.1, 1.4, 1.6, 1.8, 1.12, 2.4, 2.6, 2.8, 2.9, 2.10, 3.1, 3.2, 3.3, 3.7, 10.1, 10.5, 10.9
214	1.4, 2.3, 2.8, 3.2, 10.1	1.4, 2.3, 3.6, 10.1
224	1.4, 2.3, 3.2, 10.1	1.4, 2.3, 3.6, 10.1
229	1.1, 2.4, 3.1, 10.4	1.1, 2.5, 3.1, 10.4

Table 3-13 continued

Cosmid clone	T7	SP6
234†	1.1, 2.4, 3.1, 10.4	1.1, 2.5, 2.10, 3.3, 10.9
259	1.2, 1.9, 2.7, 3.5, 10.6, 10.11	1.2, 2.7, 3.5, 3.8, 10.6
274	1.2, 2.7, 3.5, 3.8, 10.6, 10.11	1.2, 2.2, 10.6
275	1.1, 2.5, 3.1, 10.4	1.1, 2.4, 3.1, 10.4
276	1.2, 2.7, 3.8, 10.6	1.2, 2.2, 3.2, 10.10
277	1.1, 2.5, 3.1, 10.4, 10.5	1.1, 2.4, 3.1, 10.4
395	1.1, 2.5, 3.3, 10.9	1.1, 2.5, 3.3, 3.6, 10.8
449	1.4, 1.8, 2.8, 2.9, 3.2, 10.1	1.8, 2.6, 3.2, 10.1
450	1.5, 2.1, 3.4, 10.5	1.5, 2.5, 3.4, 10.5, 10.6
452	1.1, 1.13, 2.7, 3.3, 10.7	1.1, 2.9, 3.3, 10.9
509	1.4, 1.8, 2.8, 3.2, 10.1	1.8, 2.9, 3.2, 10.1
528	1.2, 2.2, 3.2, 10.6	1.2, 2.2, 3.2, 10.10
543	1.5, 2.1, 3.8, 10.5	1.3, 2.1, 3.5, 10.2
544	1.2, 2.2, 3.2, 10.6, 10.10	1.2, 2.2, 3.2, 10.3, 10.10

* Later isolate of cosmid 57, likely a contaminant.
† Chimeric cosmids.

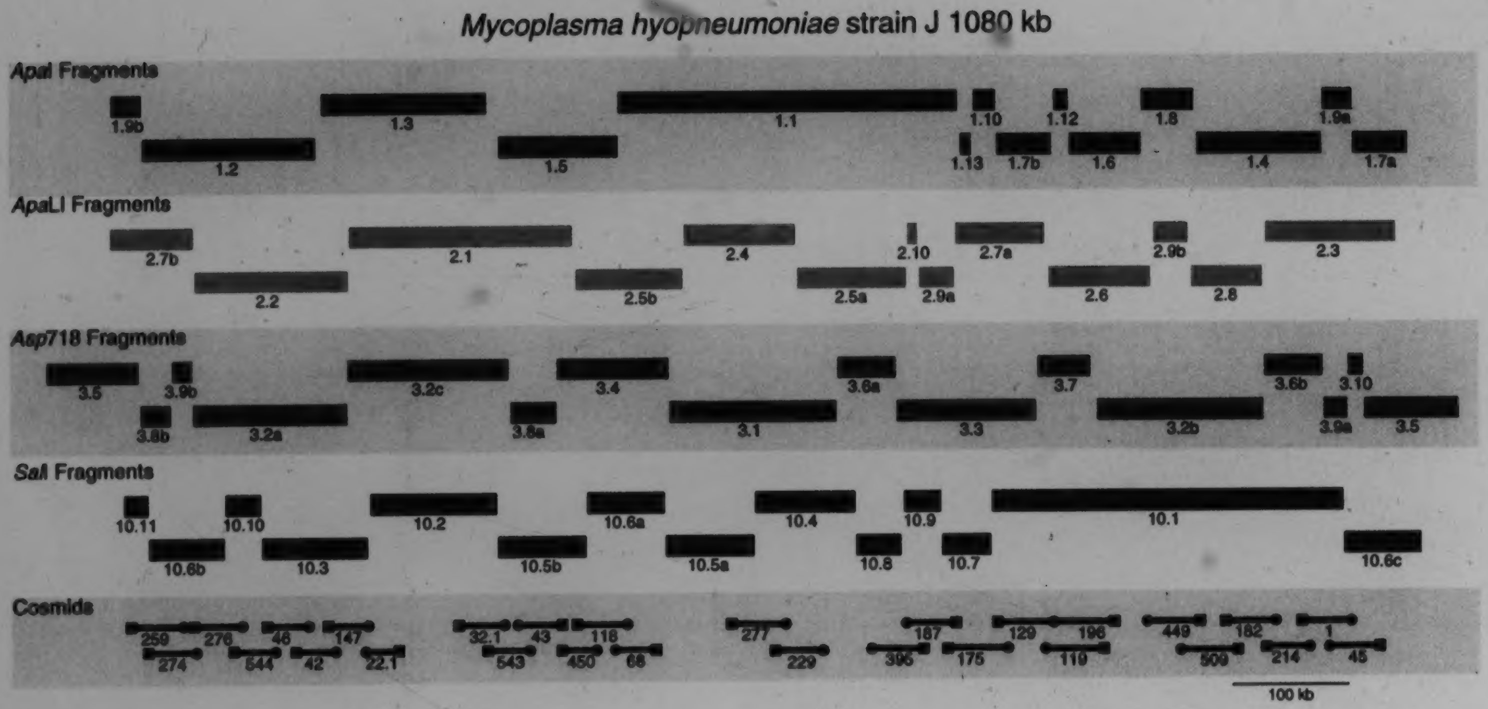
Mycoplasma hyopneumoniae strain J 1080 kb
ApaI Fragments
1.9b
1.2
1.3
1.5
1.1
1.10
1.13
1.12
1.7b
1.6
1.8
1.4
1.9a
1.7a
ApaLI Fragments
2.7b
2.2
2.1
2.5b
2.4
2.5a
2.10
2.9a
2.7a
2.6
2.9b
2.8
2.3
Asp718 Fragments
3.5
3.9b
3.8b
3.2a
3.2c
3.8a
3.4
3.1
3.6a
3.3
3.7
3.2b
3.6b
3.10
3.9a
3.5
SalI Fragments
10.11
10.6b
10.10
10.3
10.2
10.5b
10.6a
10.5a
10.4
10.8
10.9
10.7
10.1
10.6c
Cosmids
259
274
276
544
46
42
147
22.1
32.1
543
43
450
118
68
277
229
395
187
175
129
119
196
449
509
182
214
1
45
100 kb

Figure 3-15. Physical map of the *M. hyopneumoniae* genome, determined by CHEF electrophoresis of macrorestriction fragments, Southern hybridization, and cross-screening of cosmid clones. Individual restriction fragments are represented by rectangles, cosmids by horizontal lines. Locations of T7 and SP6 of the cosmid insert ends are indicated by squares and circles, respectively. Cosmid lengths are not necessarily to scale with macrorestriction fragments (see discussion).

of the restriction sites used to create the physical map, and therefore identified adjacent fragments from the same digest. This was particularly useful for the placement of smaller fragments; 1.13 and 2.10 were localized in this manner by transcripts from cosmids 452 and 234, respectively, even though the cosmids were not of use for extending contigs (234 was chimeric and 452 was redundant). Only *Apa*I fragment 1.11 could not be placed on the map. Hybridization of probes from cosmids 259 and 45 to *Apa*LI fragment 3.5, as well as its overlap with fragments 1.2, 1.6, 2.3, and 2.7 demonstrates that the *M. hyopneumoniae* chromosome is indeed circular.

Three of the cosmid transcripts, SP6-129, SP6-196, and T7-182, hybridized to a relatively large number of fragments from each digest (Table 3-12; see Figure 3-16). The actual locations of the respective cosmid ends were identified by a more intense signal from certain bands, but comparison of the other bands indicates that the probes are hybridizing to five discrete regions of the genome, identified as: (a) the region surrounding fragment 1.12 (the location of the SP6 ends of 129 and 196); (b) the overlap between fragments 1.4 and 2.8 (the location of the T7 end of 182); (c) the overlap between 2.4 and 10.5a (which enabled T7-182 to cross-screen with cosmid 277); (d) the *Apa*I site between fragments 1.6 and 1.8 (which enabled T7-182 to cross-screen with cosmid 449); and (e) fragment 2.10 (see Figure 3-17). This suggests the presence of five copies of a repetitive DNA element (designated *repJ*) which is coincidentally near one of the ends of each cosmid insert 129, 182, and 196. In fact, the *Eco*RI site separating 129 and 196 (Figure 3-14) appears to be within one of the copies of *repJ*.

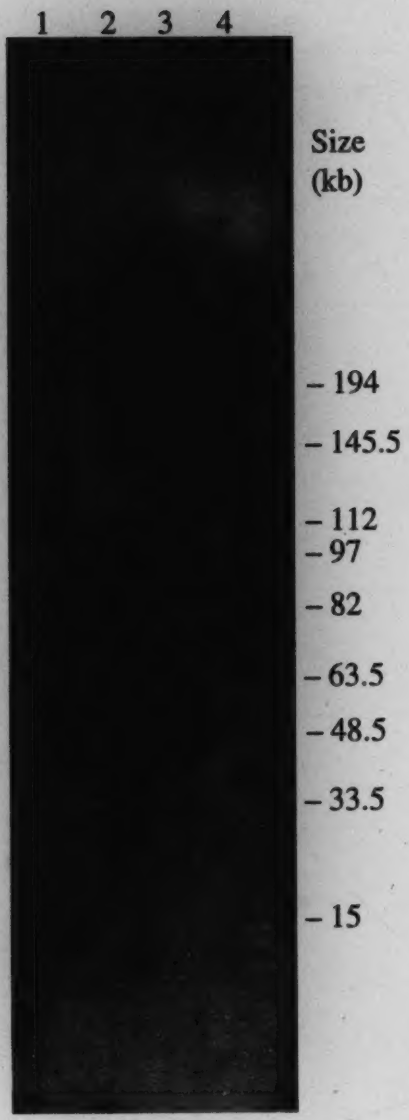
1
2
3
4
Size
(kb)
– 194
– 145.5
– 112
– 97
– 82
– 63.5
– 48.5
– 33.5
– 15

Figure 3-16. Hybridization of transcript probe SP6-129 to repetitive elements in *M. hyopneumoniae* genome. Target DNA was a Southern blot of *M. hyopneumoniae* genomic DNA digested with *Apa*I, *Apa*LI, *Asp*718, and *Sal*I (lanes 1-4, respectively) and separated by CHEF.

3.5.5 Placement of genetic markers on the map

The gene probes listed in Tables 3-1 and 3-2 were hybridized to Southern blots of CHEF separated *M. hyopneumoniae* genome digests (Table 3-14), and the locations of the genes were identified upon the *M. hyopneumoniae* physical map (Figure 3-17). The origin of chromosomal replication, as indicated by the *dnaA* gene, was located to the *Asp*718 fragment 3.8b. Two of the random *M. hyopneumoniae* plasmid clones, 13-1a and 28-1b, each hybridized to two separate locations on the *M. hyopneumoniae* chromosome. Clone 28-1b appears to be associated with two copies of *repJ*, but 13-1a is located in the overlap between fragments 2.2 and 10.6b (near the origin of replication) and in the overlap between fragments 1.8 and 2.8. The partial sequences of neither clone showed homology to any sequences in public databases, nor did they resemble each other.

Table 3-14. Hybridization of genetic probes with *M. hyopneumoniae* macrorestriction fragments. See tables 3-1 and 3-2 for identification of probes.

	Fragments hybridized			
Probe	*Apa*I	*Apa*LI	*Asp*718	*Sal*I
23S rRNA	1.2	2.7	3.5	10.6
16S rRNA	1.2	2.7		10.6
5S rRNA	1.4	2.3	3.2	10.1
adh	1.3	2.1	3.2	10.2
ctpS	1.1	2.5	3.4	10.6
dnaA	1.2	2.7	3.8	10.6
gltS	1.2	2.7	3.5	N.D.
glyT	1.4	2.3	3.6	10.1
gyrA	1.4	2.8	3.2	10.1
hsp60	1.5	2.1	3.8	10.5
ileS	1.2	2.2	3.2	10.10
IS1221	1.1	2.5	3.6	10.4
ldh	1.3	2.1	3.2	10.2
mapT	1.6	2.6	3.2	10.1
nrdF	1.5	2.1	3.8	10.5
p46	1.6	2.6	3.2	10.1
pfkB	1.5	2.1	3.4	10.5
ptsII	1.4	2.8	3.2	10.1
pyk	1.3	2.1	3.2	10.2
recA	1.4	2.8	3.2	10.1
rpoB	1.2	2.7	3.5	10.6
rpoD	1.2	2.2, 2.7	3.2	10.6
samS	1.1	2.7	3.3	10.7
secA	1.3	2.2	3.2	10.3
trpS	1.2	2.2	3.2	10.3
tuf	1.8	2.6	3.2	10.1
valS	1.2	2.7	3.8	10.6
B5-1b	1.1	2.5	3.6	10.4
6-1a	1.10	2.7	3.3	10.1

Table 3-14 continued.

	Fragments hybridized			
Probe	*Apa*I	*Apa*LI	*Asp*718	*Sal*I
B7-1b	1.1	2.5	3.6	10.4
B11-1b	1.6	2.6	3.2	10.1
13-1a	1.2, 1.8	2.2. 2.8	3.2	10.1, 10.6
B14-1b	1.4	2.8	3.2	10.1
B26-2a	1.2	2.2	3.2	10.10
B28-1b	1.1, 1.8	2.4, 2.6	3.1, 3.2	10.5, 10.1

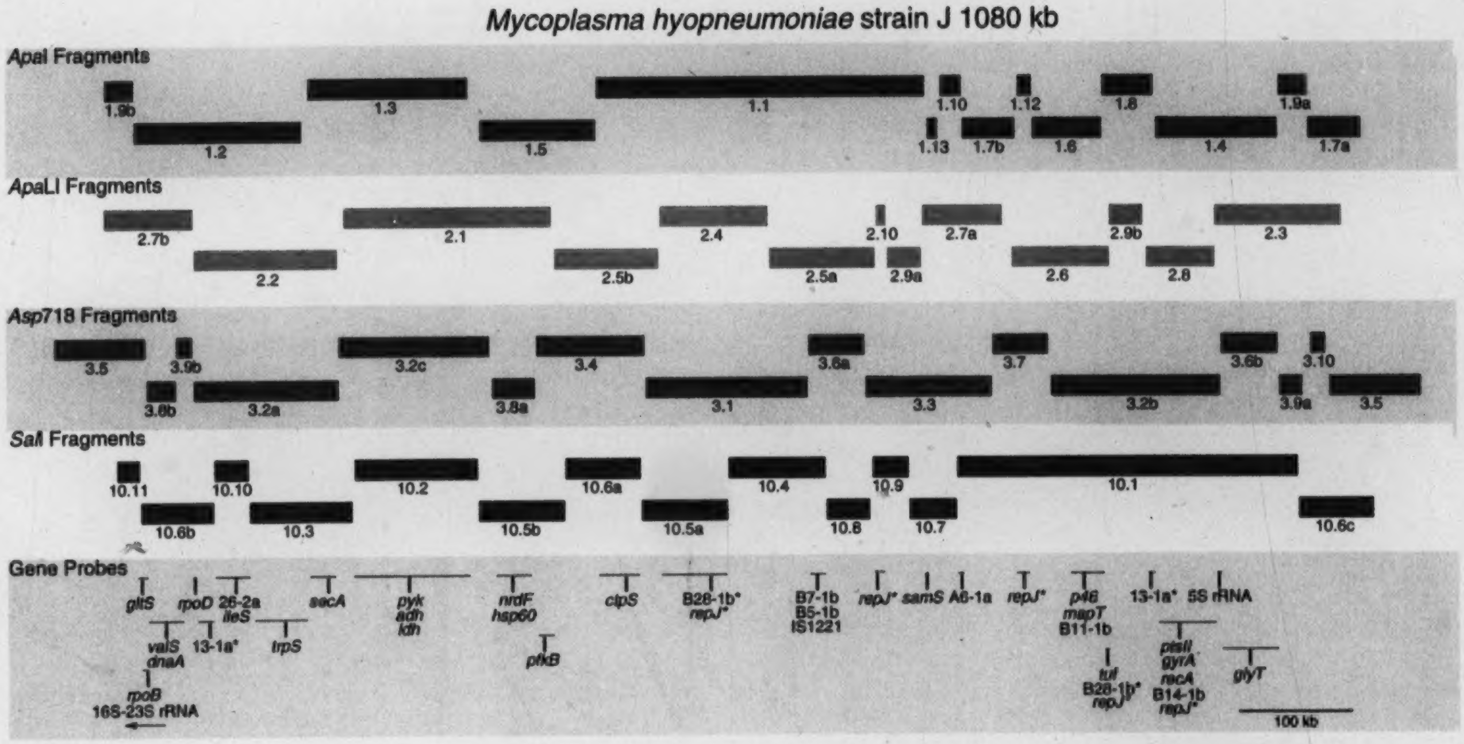
Mycoplasma hyopneumoniae strain J 1080 kb
ApaI Fragments
1.9b
1.2
1.3
1.5
1.1
1.10
1.13
1.7b
1.12
1.6
1.8
1.4
1.9a
1.7a
ApaLI Fragments
2.7b
2.2
2.1
2.5b
2.4
2.5a
2.10
2.9a
2.7a
2.6
2.9b
2.8
2.3
Asp718 Fragments
3.5
3.9b
3.6b
3.2a
3.2c
3.8a
3.4
3.1
3.6a
3.3
3.7
3.2b
3.6b
3.10
3.9a
3.5
SalI Fragments
10.11
10.6b
10.10
10.3
10.2
10.5b
10.6a
10.5a
10.4
10.8
10.9
10.7
10.1
10.6c
Gene Probes
gltS
rpoD
26-2a
ileS
valS
dnaA
13-1a*
rpoB
16S-23S rRNA
trpS
secA
pyk
adh
ldh
nrdF
hsp60
pfkB
clpS
B28-1b*
repJ*
B7-1b
B5-1b
IS1221
repJ*
samS
A6-1a
repJ*
p46
mapT
B11-1b
tuf
B28-1b*
repJ*
13-1a*
5S rRNA
ptsII
gyrA
recA
B14-1b
repJ*
glyT
100 kb

Figure 3-17. Physical and genetic map of the *M. hyopneumoniae* genome, as determined by CHEF electrophoresis of macrorestriction fragments and Southern hybridization. Individual restriction fragments are represented by rectangles, horizontal lines are ambiguity bars covering the area within which each element is located. Multiple elements under one ambiguity bar are not listed in any particular order. Refer to Tables 3-1 and 3-2 for identification of gene probes. Where possible, markers are labelled with their respective gene symbols. Repetitive elements are marked with asterisks (*). Arrow indicates direction of transcription of rRNA operon.

4. DISCUSSION

4.1 The *M. hyosynoviae* 16S rRNA gene

PCR primers complementary to 16S rRNA terminal sequences conserved in mollicutes (Robertson et al. 1993) were capable of amplifying the 16S rRNA gene from *Mycoplasma hyosynoviae* genomic DNA. Sequencing of cloned products from two amplification reactions showed that there were five point differences between the two sequences. This small difference between the sequences (< 0.5%) makes it unlikely that one of the products came from a cultural contaminant, as the two sequences were far more similar to each other than to any other characterized mycoplasma 16S rRNA sequences. The possibility that there are two slightly different 16S rRNA genes present in the *M. hyosynoviae* genome was eliminated by the presence of only a single hybridizing band when digested genomic DNA was probed with labelled oligonucleotides (Figure 3-4). The presence of a single copy of the 16S rRNA gene in the *M. hyosynoviae* genome agrees with the conclusion of Amikam *et al* (1984) that mollicutes carry only one or two sets of rRNA genes. Possession of a low number of rRNA genes by an organism has been correlated with a slow growth rate (Fegatella et al. 1998; Suzuki et al. 1987), a characteristic which is common among mycoplasmas.

The most likely explanation for the differences seen between the two clones is that several nucleotides had been misincorporated by Taq DNA polymerase during PCR. Alignment of the sequences obtained from pR1 and pR3 with 16S rRNA sequences from other mycoplasmas showed that each of these differences occurred at positions that were conserved among all other mycoplasmas, and one of the clones always had the agreeing nucleotide. The lack of a proofreading system in Taq (Tindall and Kunkel 1988) results in such errors which, although not very frequent (4 bases out of 1499 in pR3, one in pR1, after 30 cycles of amplification), may affect phylogenetic analysis of very closely related molecules. Keeping the number of amplification cycles to a minimum and sequencing the products of more than one reaction would prevent erroneous conclusions from being drawn. Direct sequencing of PCR products, using fluorescently labelled dideoxynucleotides or primers (White et al. 1990), could identify subpopulations of products within a PCR reaction and help to identify errors. The use of a proofreading heat-stable DNA polymerase (such as Pfu or Vent polymerase) in the PCR reaction could prevent misincorporation of nucleotides, but attempts to use Pfu to amplify the *M. hyosynoviae* 16S rRNA gene gave a second 1200 bp product. This product hybridized with radiolabelled oligonucleotide GWS 6 in a Southern blot (not shown) suggesting that it was a truncated 16S rRNA gene, and this was confirmed by partial sequencing. Again, the failure of oligonucleotide probes to detect a second gene in *M. hyosynoviae* genomic DNA, and the fact that Taq polymerase consistently amplified only a single, full length product indicated that this was an artifact of the amplification reaction. It is possible that the considerable secondary structure present in rRNA sequences may be difficult for Pfu to properly replicate.

4.2 Phylogenetic analysis of *M. hyosynoviae* and *M. hyopharyngis*

The demonstration in this study that there is no close phylogenetic relationship between either *M. hyosynoviae* or *M. hyopharyngis* and the porcine members of the *M. hyorhinis* group (including *M. hyopneumoniae* and *M. flocculare*) is not entirely surprising, given the metabolic differences between the organisms. Both *M. hyosynoviae* and *M. hyopharyngis* are capable of metabolizing arginine but not glucose, as are their closest relatives (Weisburg et al. 1989), whereas the opposite is true of the *M. hyorhinis* group. A previous study (Johansson et al. 1992) showed that a 27 base oligonucleotide probe specific for *M. hyorhinis* 16S rRNA showed weak cross-hybridization with dot blots of total nucleic acid from *M. hyosynoviae* which was not seen with other porcine mycoplasmas. The complementary sequence to this probe is not present in the *M. hyosynoviae* 16S rRNA molecule with fewer than 12 mismatches (data not shown). It is possible that the hybridizing sequence is present (by chance) elsewhere on the *M. hyosynoviae* genome and that the probe detected *M. hyosynoviae* DNA.

Both of the nearest characterized relatives of *M. hyosynoviae*, *Mycoplasma orale* and *M. salivarium*, are commonly isolated from the human oropharynx and are considered part of the normal human microflora (Krause and Taylor-Robinson 1992; Somerson and Cole 1979). *Mycoplasma lipophilum*, the closest known relative of *M. hyopharyngis*, is also part of the human oral microflora (Krause and Taylor-Robinson 1992). The close relationship of *M. hyosynoviae* and *M. hyopharyngis* to human mycoplasmas in particular can probably be explained by the relatively intense investigation of the human microflora, and related species of mycoplasmas may not yet have been characterized in animals other than swine. A less likely alternative is the possibility of horizontal transmission from one host to the other since the domestication of swine by humans, followed by divergence due to specialization to the new host. Even the accelerated pace of evolution in mycoplasmas (Weisburg et al. 1989) is unlikely to have resulted in such a rapid genetic change as to eliminate antigenic cross-reactivity between the human and porcine mycoplasmas in a period of perhaps ten thousand years. Studies to estimate divergence times between mycoplasma species using molecules such as 16S rRNA as molecular clocks have not been performed. Examination of non-domesticated relatives of swine will likely reveal organisms closely related to *M. hyosynoviae* and *M. hyopharyngis*, much as *M. salivarium* is commonly found among non-human primates (Somerson and Cole 1979).

The use of other conserved genes such as those encoding the elongation factor Tu (Kamla et al. 1996; Schneider et al. 1997), the heat shock protein DnaK (Falah and Gupta 1997), conserved ribosomal proteins (Gundersen et al. 1996), and the 16S-23S rRNA spacer region (Smart et al. 1996) as phylogenetic markers has also been effective in determining relationships among mycoplasmas (particularly unclassified phytoplasmas) and, in cases where comparisons have been made, generally agree with the 16S phylogenies. An exception is a study done using the EF-Tu gene (Kamla et al. 1996) which showed a close relationship between *M. pneumoniae* and *M. genitalium* that was not seen in a 16S-based tree they presented. However, a previous study (Maniloff 1992b) and a phylogenetic analysis done in this study (data not shown) indicate that the *M. pneumoniae* and *M. genitalium* 16S sequences are indeed very similar, suggesting that the sequence for the *M. genitalium* 16S used by Kamla et al was incorrect. On a cautionary note, a recent paper describing the inactivation of all rRNA operons in a strain of *E. coli* and complementation with rRNA genes from *Salmonella typhimurium* and *Proteus vulgaris* (Asai et al. 1999) suggests that even a highly co-evolved system such as the translation apparatus may be susceptible to horizontal transfer of genes. Although such transfers in nature have not been observed, it may be prudent to re-evaluate the use of 16S rRNA sequences as the sole molecular determinant of the history of microorganisms.

4.3 Mapping the *M. flocculare* genome

In preliminary experiments of this study using FIGE (data not shown) the phenomenon of band scrambling was observed, in which larger bands (>400 kb) from the λ concatamer molecular weight marker sometimes migrated ahead of some of the larger *M. flocculare* digest bands (220 kb), making identification of some bands difficult and casting doubt upon the sizes derived. A physical map of the *M. flocculare* chromosome constructed using field inversion gel electrophoresis (FIGE) had previously been published (Huang and Stemke 1992) but inconsistencies with the data obtained in this study with regard to the size and number of restriction fragments visible after electrophoresis prompted the reconstruction of the map using the same restriction enzymes, but separating the fragments by CHEF electrophoresis. In general, sharper bands and more consistent sizing of the bands were observed using CHEF.

It was usually seen that some genomic DNA remained in the agarose plugs placed in the gel wells after completion of the CHEF run, as seen in Figures 3-6, 3- 9, and 3-10, and is apparently not digested at all by the restriction enzymes. The reason for this is not known. The restriction digests normally went to completion, as evidenced by the reproducibility of digest patterns and lack of bands corresponding to partial digest products, except for the two partial digest products seen in *SalI* digests of *M. flocculare*, which were also seen in the previous study (Huang and Stemke 1992). It is possible that the DNA remaining in the wells was trapped inside mycoplasma cells that were not completely degraded during the treatment of the agarose plugs with SDS and proteinase K, although lengthening this treatment did not substantially alleviate the problem. Precipitated media components which were pelleted during culture harvests could have prevented complete digestion of cells. At any rate, this phenomenon did not adversely affect the results since enough DNA was digested to visualize on the gel after separation. In addition, the genomic DNA left in the wells did transfer to the nylon membranes during Southern blotting and acted as an accurate origin point for the measurement of band migration, since probes would hybridize to it as well as to the digest bands (Figure 3-7).

The total size of the *M. flocculare* ATCC 27716 genome (~900 kb) obtained by summing the sizes of all fragments from each digest agrees with that determined by

Robertson et al. (1990) of the unfragmented chromosome, although it is shorter than that reported for a digested *M. flocculare*ATCC 27399 chromosome by Frey et al (1992). Both strains are derived from clones of the type strain Ms 42 (Cote 1992), and the differences between them are possibly due to different passage numbers and growth conditions leading to chromosomal rearrangements (Frey et al. 1992).

A physical map of the *M. flocculare* genome (Figure 3-8) was constructed by determining overlaps between CHEF-separated macrorestriction fragments. A total of 29 restriction sites (giving 10 *Apa*I, 8 *Asp*718, and 10 *Sal*I fragments) were characterized in the 900 kb genome, giving a mean resolution of approximately 32 kb, although since only single-enzyme digests were used in this study the precise extent of overlaps between fragments was not determined. However, the map obtained is of sufficient resolution to determine the gross genetic arrangement of the *M. flocculare* genome and compare it to that of *M. hyopneumoniae* (see below). The *Apa*I site separating fragments 7.6 and 7.3, and the *Asp*718 site separating 8.1 and 8.4 are apparently very close together (leaving very little overlap between fragments), because no complementary hybridization was seen among those fragments. The circularity of the *M. flocculare* genome was confirmed by probing with band 9.5 (consisting of three separate *Sal*I fragments) which linked 7.3 to 7.6 and 8.1 to 8.4 with a fragment designated 9.5c.

Certain hybridization patterns occasionally occurred during probing which did not agree with the final map. For instance, when using band 9.5 as a probe, fragments 7.5 and 8.3 were hybridized; conversely, both probes 7.5 and 8.3 hybridized to 9.4 and 9.5. In such cases, fragment ordering on the map was based on the relative strength of the hybridization signal from each band. These spurious signals could be a result of contamination with other DNA fragments during the band isolation procedure, due to imperfect separation of fragments during CHEF. Another likely explanation is the presence of uncharacterized repetitive elements in the *M. flocculare* genome. Several such elements were discovered in the *M. hyopneumoniae* genome (see below), although no further investigation of repetitive elements in the *M. flocculare* genome was performed.

Comparison of this CHEF-based physical map of the *M. flocculare* genome with that previously obtained by FIGE (Huang and Stemke 1992) revealed some inconsistencies aside from minor differences in estimated fragment lengths which can be attributed to the different electrophoretic methods used. The ordering of *Asp*718 fragments in both maps was identical, although the similarly sized fragments 8.4 and 8.5 (my notation) were resolved as a doublet band by FIGE. More marked differences were seen with the *Apa*I and *Sal*I digests. Huang had noted only nine *Apa*I fragments, as opposed to my ten, but examination of the photograph of the FIGE gel (Figure 1a, Huang and Stemke, 1992) suggests that a doublet band (corresponding to my fragments 7.5 and 7.6, which were separately resolved by CHEF) was misinterpreted as a single restriction fragment in the earlier study. Ordering of the *Apa*I fragments which overlapped with the largest *Asp*718 fragment also differed between the two maps. Two more bands appear to be missing from the *Sal*I digest from the earlier study, although the 110 kb band (corresponding to my fragment 9.3) appeared as a doublet. This suggests that at least one *Sal*I restriction site on the map derived in this study could have arisen through a point mutation in the genome. Although the same strain of *M. flocculare* was used in both studies, differences in the number of broth passages that each respective stock culture had undergone could result in different PFGE restriction fragment patterns, as had been previously noted in *M. hyopneumoniae* (Frey et al. 1992).

Overall, the PFGE map of the *M. flocculare* chromosome determined in this study can be considered as an improvement over the earlier map because it was constructed using the CHEF electrophoresis system, which allows for more accurate comparison of DNA fragment sizes without generation of band-scrambling artifacts. In addition, hybridization data was obtained using all of the CHEF-separated restriction fragments as probes (26 eluted gel bands in total), as opposed to only 13 *Apa*I, *Asp*718, and *Sal*I fragments (plus 3 other restriction fragments) used as probes by Huang.

Placing genetic markers upon the deduced physical map of the *M. flocculare* genome did not reveal any inconsistencies in the ordering of the fragments, insofar as the overlaps between macrorestriction fragments agreed with the fragments which hybridized to each of the gene probes. Not all of the gene probes tested could be placed upon the *M. flocculare* genome map. Since most of the probes originated from *M. hyopneumoniae* (either by PCR amplification or random cloning), this can be accounted for by two explanations: the gene is not present in the *M. flocculare* genome; or the *M. flocculare* homolog differs enough from the gene probe to prevent detection under the stringency conditions of the particular hybridization and subsequent washings. (PCR amplification of gene probes using degenerate primers was only performed using *M. hyopneumoniae* genomic DNA, since optimization for each primer/template combination required the adjustment of a number of variables, including salt and Mg^{2+} concentration, pH, and annealing temperature.) Although efforts were made to balance stringency and sensitivity, very often lowering stringency resulted in unacceptable nonspecific binding of the probe to other bands. In the instance of both the P46 and elongation factor Tu genes, nonspecific hybridization to nearly all the *M. flocculare* restriction fragments was seen, but the relative intensity of the hybridization signal from fragment 8.6 was enough to localize them both to the same 45 kb region of the *M. flocculare* genome. Increasing the stringency of the washes in these cases resulted in the loss of any hybridization signal. A previous study (Stemke et al. 1985) has shown that *M. hyopneumoniae* and *M. flocculare* only demonstrate approximately 10% heterologous binding under moderately stringent washing conditions (2X SSPE with 0.1% SDS, at 45 °C), which suggests that only the most well-conserved genes can be detected with heterologous probes.

Obviously, the functional RNA gene probes (the rRNAs and glycine-tRNA) have highly conserved sequences, permitting hybridization. Because there happens to be an *Apa*LI site between the 16S and 23S genes (Table 3-6), the direction of transcription of that operon (i.e., away from the origin of chromosomal replication region, *dnaA*) is known. As previously reported, the 5S gene is distantly located on the chromosome (Huang and Stemke 1992). Several of the genes placed on the *M. flocculare* map are major antigens from *M. hyopneumoniae*. Recombinant proteins from the *p46*, *ldh*, and *nrdF* genes from *M. hyopneumoniae* all elicit a strong immune response (Fagan et al. 1996; Futo et al. 1995b; Strasser et al. 1991), although antibodies against P46 and Ldh were indicated as very species specific for *M. hyopneumoniae* against whole protein cell preparations from different porcine mycoplasma, including *M. flocculare*. This could indicate that particular epitopes are not present in the *M. flocculare* proteins, or that they are differently expressed in the two species.

4.4 Mapping the *M. hyopneumoniae* genome

4.4.1 CHEF analysis

Comparison of CHEF separated restriction digest fragments between *M. hyopneumoniae* strain J and *M. hyopneumoniae* ATCC 25095 (Figure 3-9) revealed a striking difference between the two strains, although a number of fragments were conserved. Such conserved restriction fragments were not observed when compared to *M. flocculare*. Chromosomal heterogeneity between strains of *M. hyopneumoniae* has been previously demonstrated by Frey et al. (1992) in a study of ten strains. Genetic heterogeneity has also been shown between *M. hyopneumoniae* isolates by arbitrarily primed PCR (Artiushin and Minion 1996). Whether such differences reflect chromosomal rearrangements or merely single base pair mutations within restriction sites (or in the case of AP-PCR, primer binding sites) is not known; further chromosomal analysis of other strains will be required. Comparative PFGE mapping of *M. mycoides* strains (Pyle et al. 1990) and *M. hominis* strains (Ladefoged and Christiansen 1992) has revealed that most heterogeneity between strains involved restriction fragment lengths, although gene rearrangements had occurred in some cases.

The genome size of 1080 kb (averaged from totals of restriction fragments presented in Tables 3-7 through 3-10) for *M. hyopneumoniae* strain J agrees with previous estimates of *M. hyopneumoniae* genome sizes determined by PFGE (Frey et al. 1992; Huang and Stemke 1992; Robertson et al. 1990).

4.4.2 Construction of a physical and genetic map

Constructing a physical map of the *M. hyopneumoniae* genome by probing Southern blots of CHEF gels with isolated macrorestriction fragments was not as straightforward as with *M. flocculare*. Greater numbers of fragments, the more frequent occurrance of multiple similarly-sized fragments in a single band, and repetitive elements in the genome (see below) prevented the assembly of a complete map using only this method. The simultaneous assembly of an overlapping cosmid library of *M. hyopneumoniae* strain J permitted the combination of these two approaches to construct the genomic map.

The cross-screening procedure (Locke et al. 1996) was used to assemble the cosmid library into contigs of overlapping clones. Ideally, this method would be well suited to the analysis of small genomes such as those of mycoplasmas, as it permits the rapid determination of overlap and orientation (with regard to T7 and SP6 ends of inserts) among up to 45 clones simultaneously. Reciprocal rounds of screening among five groups of about 40 cosmids permitted identification of five contigs and a number of unlinked orphan cosmids (Table 3-11) covering approximately half of the *M. hyopneumoniae* genome.

However, assembly of a complete overlapping library could not be achieved, due to particular characteristics of the *M. hyopneumoniae* genome and limitations of the cosmid library itself. The presence of previously uncharacterized repetitive elements in the genome led to misidentification of overlaps between cosmids that were later shown to be widely separated. For example, cosmid 182 (which contains a *repJ* element) would hybridize with a number of cosmids which upon *Eco*RI digest analysis did not share any common fragments. Chimeras could not be identified as such and could potentially lead to incorrect contigs. Furthermore, the relationship of the contigs to each other and size of gaps between them could not be determined. For these reasons, when cross-screening of the second batch of 350 cosmids was performed, identified cosmids (including those already included in contigs) were tested by hybridization of their T7 and SP6 transcript probes to Southern blots of *M. hyopneumoniae* CHEF separated digests.

The advantages of this were twofold. Firstly, it facilitated the rapid assembly of the macrorestriction fragment map (Figure 3-15). Not only did each individual probe identify overlapping fragments (and if the transcript probe happened to include one of the rare restriction sites used in the digests, identify adjacent fragments), but the transcripts from the opposite end of the insert and from overlapping cosmids would also help to identify neighboring fragments. The completed map contains 56 macrorestriction fragments and has a mean restriction site resolution of approximately 20 kb.

Secondly, the positions of contigs and individual cosmids as well as gaps in the assembled library could be determined. Chimeric cosmids (e.g. cosmids 153, 167, and 181) were easily identified by their respective transcripts hybridizing to different regions of the completed map. Through this process and further cross-screening, 28 cosmids from the library were determined to comprise the minimum overlapping set for five contigs. Agarose gel analysis after *Eco*RI digestion of the cosmids was used to determine the size of each cosmid and the extent of overlap between neighboring clones (Table 3-13) and the total coverage of the *M. hyopneumoniae* genome was calculated at 728 250 bp. This may underrepresent the actual extent of coverage, since similarly sized (but unshared) fragments in overlapping cosmids may have been counted as being part of the overlaps. The cosmids themselves do not appear to have undergone obvious deletions (with the exception of 22.1) since the size of each of them (insert length plus 5.2 kb vector) falls within the acceptable 37-52 kb range for λ packaging extracts (DiLella and Woo 1987), although most of them do fall near the lower end of this range. This is probably due to the quality of the partial genomic digests used.

Probing Southern blots of CHEF gels with genetic markers was not specifically used to order fragments on the macrorestriction map but did confirm the overlaps between fragments determined by use of the cosmid transcript probes. A total of 35 markers were placed on the map (Figure 3-17). As noted previously, the 5S rRNA gene is separated from the 16S-23S operon in this species (Huang and Stemke 1992; Taschke et al. 1986). Interestingly, the gene for the *M. hyopneumoniae* ciliary adhesin protein (*adh*) which may be a pathogenic factor in this organism (Hsu and Minion 1998) maps to within approximately 100 kb of the genes for lactate dehydrogenase and nucleotide reductase, both strongly antigenic proteins. Hsu and Minion (1998) described an open reading frame encoding a protein of 102 kDa which is part of an operon with *adh*, and furthermore may be present in four copies in the same region. This suggests a cluster of genes which may be involved in the pathology of *M. hyopneumoniae* and furthermore could provide antigenic variation through recombination among the repeated genes (Dybvig and Voelker 1996).

A puzzling early result was that the PCR product used to probe for the 16S rRNA gene did not hybridize to an *Asp*718 fragment on Southern blots of *M. hyopneumoniae* CHEF gels. Examination of the 16S and 23S rRNA gene sequences of *M. hyopneumoniae* (GenBank accession numbers E02783 and X68421, respectively) revealed that there is an *Asp*718 recognition site (GGTACC) present in each gene, and that these sites are conserved in the 16S and 23S genes of *M. flocculare* (accession number L22210). Since the two genes are closely linked (Stemke et al. 1994a; Taschke et al. 1986), digestion of chromosomal DNA in either organism would result in an *Asp*718 fragment approximately 3.5 kb in length, which is smaller than the resolution limit of the CHEF programs used in this study and would have run off of the gels. (The smallest molecular weight marker visualized on CHEF gels was 9.4 kb.) This did not prevent detection of the 23S rRNA gene on fragments 3.5 and 8.8, as the probe from plasmid clone 9-1b hybridizes to a region of this gene outside of the *Asp*718 sites (Figure 4-1). However, hybridization with the remaining portion of the 16S gene (490 bp) should have occurred in *M. hyopneumoniae* as it did with fragment 8.4 in *M. flocculare*.

Therefore, there must be a third *Asp*718 site shortly upstream of the 16S-23S operon (several kb or less) in *M. hyopneumoniae*. This site defines one end of fragment 3.8b, resulting in another short fragment containing the rest of the 16S gene which also ran out of the gel during CHEF electrophoresis. This site may be conserved in other strains of *M. hyopneumoniae*, as the 16S gene probe did not hybridize to an *Asp*718 fragment of strain ATCC 25095 upon Southern analysis either (data not shown). Assuming then that there are two short *Asp*718 fragments which contain the entire 16S rRNA gene between fragments 3.5 and 3.8b, the direction of transcription of this operon is away from the origin of replication (marked by the *dnaA* gene). In addition, since the *rpoB* gene is also located on fragment 3.5, it must be downstream of the rRNA genes.

4.4.3 Gaps in the overlapping cosmid library

Assuming that the *M. hyopneumoniae* genome size is 1080 kb (as determined by CHEF) and that the average cosmid insert length is 35 kb, a random (exponential) distribution of 100 cosmids should have covered greater than 95% of the genome, and 99% coverage should have been obtained with 150 cosmids. Over three times this number of cosmids were screened by cross-screening alone in this study, yet the total genome coverage obtained by all contigs (Table 3-13) was only about 728 kb, or 67%. This low value may be at least partly due to overestimation either of the extent of overlap between cosmids (due to similarly sized fragments in neighboring cosmids) or of genome size by PFGE (see discussion of this topic below), but the fact remains that there are five gaps in the overlapping library which cannot be accounted for on a purely statistical basis.

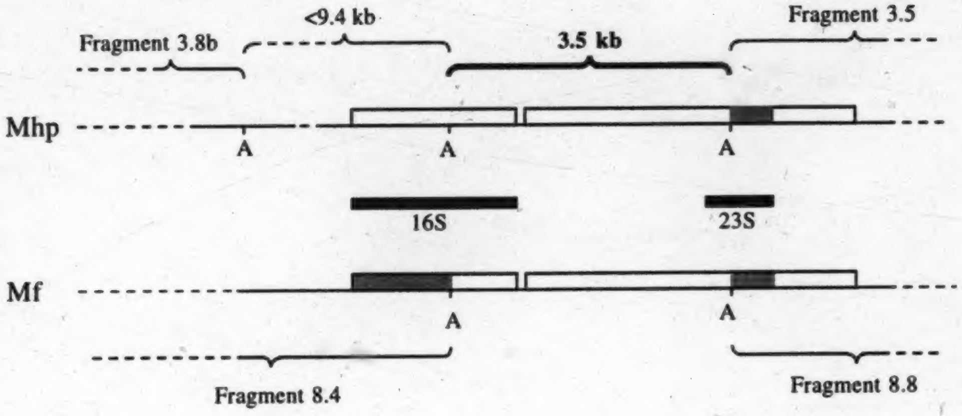
Fragment 3.8b
<9.4 kb
3.5 kb
Fragment 3.5
Mhp
A
A
A
16S
23S
Mf
A
A
Fragment 8.4
Fragment 8.8

Figure 4-1. *Asp*718 maps of 16S-23S rRNA gene regions in *M. hyopneumoniae* (Mhp) and *M. flocculare* (Mf). *Asp*718 sites (A) are labelled and the portions of the genes hybridizing with the 16S and 23S rRNA probes in Southern analysis of CHEF gels are indicated by shading.

Agarose gel electrophoresis of *M. hyopneumoniae* genomic DNA completely digested by *Eco*RI (not shown) showed a large number of restriction fragments less than 10 kb long, but a few fragments greater than 10 kb in size were also seen. The largest of these (17.5 kb) is present in cosmid insert 182, but several fragments from 13 to 16 kb long are not seen in any of the cosmid clones. If several of these fragments are located closely to each other in the genome and were not separated during the partial digestion used to create the library, the resulting fragments may have been too large to package in the λ extracts after ligation with pcosRW2, resulting an a gap in that region of the genome. Gaps of this sort could be filled by screening a second cosmid library created with genomic DNA partially digested by a more frequently cutting restriction enzyme such as *Sau*3A.

It is possible that certain gene products encoded in the gap regions are toxic to the *E. coli* host and cosmids carrying them are therefore not propagated after transfection. Transcription signals found in mycoplasmas are similar to those in other eubacteria (Dybvig and Voelker 1996; Gafny et al. 1988) and a Shine-Delgarno ribosome binding sequence is located upstream of the initiation codons of most (but not all) mycoplasma genes (Razin et al. 1998). Cloned mycoplasma genes have been expressed in *E. coli* (Muto et al. 1992) although most products are truncated due to the use of UGA to encode tryptophan in mycoplasmas, while *E. coli* reads it as a stop signal. In fact, either site-directed mutatgenesis of all TGA codons (Futo et al. 1995b; King et al. 1997) or use of opal-supressor strains of *E. coli* (Renbaum et al. 1990; Smiley and Minion 1993) is required for full-length protein expression from most mycoplasma genes. Regardless, partial proteins could still be disruptive to *E. coli* metabolism, particularly if they are homologous to certain host proteins.

Cosmid insert instability is a well-known drawback of the use of these vectors (Kim et al. 1992) and is probably the main culprit behind the gaps in the overlapping library. The insert from cosmid 22.1, which encroaches upon an uncloned region (Figure 3-15), is less than 20 kb in length, almost certainly as a result of partial deletion. On the opposite side of the same gap, cosmid 32.1 was observed to have lost several *Eco*RI fragments in a later culture passage, but it was possible to recover the full length insert from stocks. Both of these cosmids were identified by probing colony blots of the *M. hyopneumoniae* cosmid library with transcript probes from the ends of their respective contigs. Colonies identified by probes from the ends of other contigs invariably contained cosmids which had undergone drastic deletions with only small DNA species remaining. These results strongly suggest that the sequences in the gap regions are quite unstable in the *E. coli* host and likely cannot be cloned in cosmid vectors.

Two other overlapping clone libraries of mycoplasma genomes (*M. pneumoniae* and *M. genitalium*) also reported gaps which could not be filled using cosmid clones (Lucier et al. 1994; Wenzel and Herrmann 1989). In both cases the gaps were bridged through the use of phage λ based cloning vectors. This is probably the best strategy to be followed in filling the gaps in the *M. hyopneumoniae* library. In order to enrich the λ library for sequences which have not already been cloned, it may be most practical to construct it from DNA fragments isolated from preparative CHEF gels. As seen in Figure 3-15, macrorestriction fragments 1.1 (containing the gaps between cosmids 146-277, and 229-395), 1.3 (gap 147-32.1), 3.5 (gap 45-259), and 3.2 (gaps 147-32.1 and 196-509, although part of contig I would also be represented by fragment 3.2a) could be isolated from a CHEF gel run in low melting point agarose, pooled, and partially digested into appropriately sized fragments by *Sau*3A or another frequently cutting restriction enzyme before cloning into a λ vector. Probing plaque lifts of this library with transcripts from contig ends would extend the contigs, although the size of the gaps would probably require further rounds of screening with the isolated λ clones.

An alternative would be the use of a fosmid type cloning vector (Kim et al. 1992) which carries λ *cos* sequences and an F plasmid origin of replication. This allows cloning of cosmid-sized DNA fragments but limits the copy number of the resulting plasmid in the host. Marked improvements in clone stability have been noted in this vector, presumably due to reduced recombination between multiple copies (Kim et al. 1992), and the constant copy number of the vector (1-2 per cell) would reduce segregation selection for deleted inserts (Fonstein and Haselkorn 1995). A drawback, of course, would be a substantial decrease in the amount of DNA isolated (at least 10-fold) during each fosmid culture preparation compared to cosmids.

4.4.4 Repetitive elements

This study revealed three different types of repetitive element in the *M. hyopneumoniae* genome. Plasmid clones 13-1a and B28-1b, randomly cloned fragments of *M. hyopneumoniae* genomic DNA, each hybridize to two locations on the *M. hyopneumoniae* chromosome (Figure 3-17). Partial nucleotide sequences from both clones show no homology to sequences in public databases. These elements may be portions of duplicate or homologous genes; however, both copies of the B28-1b element mapped closely with the third type of repetitive element, which was designated *repJ*.

The *repJ* elements were identified by virtue of being present at or near one end of three cosmid clone inserts. Transcript probes from each of these cosmids hybridized to five distinct locations on the *M. hyopneumoniae* chromosome. It is possible that more than five copies exist, if two or more are located closely together and cannot be resolved on the PFGE map. These repeats all appear to reside in one half (~500 kb) of the chromosome, which contains the chromosomal replication terminus (i.e. approximately half way around the chromosome from *dnaA*).

Harasawa *et al* (1995) have described a 4.2 kb repetitive element in *M. hyopneumoniae* strain VPP11, of which eight copies are present in the genome of that strain. The element contains 270 bp direct repeats at either terminus and also carries three open reading frames to which no function has been assigned. This suggests that it may comprise a mobile DNA element of some kind, although its structure is unlike any transposon or insertion sequence found in bacteria. Whether it is homologous to *repJ* is not yet known; subcloning and sequencing of the ends of cosmids 129, 196, and 182, or probing with the VPP11 element will confirm whether or not this is the case. Attempts to directly sequence the SP6 ends of cosmids 129 and 196, and the T7 end of 182 during this study were not successful. Sequences obtained from plasmid clone B28-1b, which maps closely to two of the five *repJ* copies (Figure 3-17), do not bear any resemblance to the VPP11 element.

It is interesting to note that the two regions of the *M. hyopneumoniae* genome to which clone B28-1b hybridizes (Figure 3-17) correspond to gaps in the overlapping cosmid library (Figure 3-15). This could signify that the entire gene or genetic element of which B28-1b is a part is unstable in *E. coli*. Alternatively, homologous recombination between the two sites (involving either the B28-1b elements or the two *repJ* elements associated with B28-1b) may have occurred in the culture harvested to construct the genomic library, resulting in an inversion of the region flanked by these two sites. If true, this would lead to apparently "chimeric" cosmids with T7 and SP6 ends hybridizing to these sites; however, no such chimeras were identified.

4.5 Comparative genetic mapping of *M. hyopneumoniae* and *M. flocculare*

4.5.1 rRNA genes

As noted previously, in both *M. hyopneumoniae* and *M. flocculare* the 5S rRNA gene is separated from the 16S and 23S rRNA genes, which are closely linked and transcribed from the same promoter (Stemke et al. 1994a; Taschke and Herrmann 1986; Taschke et al. 1986). The two rRNA operons are separated by approximately 150 kb in *M. hyopneumoniae* but by up to 200 kb in *M. flocculare* due to the genomic rarrangements involving the regions around both the 5S and 23S genes (see below). This separation of rRNA genes is unconventional, since in most bacteria all three are normally transcribed as a single operon in the order 16S-23S-5S, to be later processed into the three separate rRNA species (Condon et al. 1995); however, other mycoplasma species also have unusual rRNA gene arrangements. *M. gallisepticum* has one conventional rRNA operon and a second set of rRNA genes in which the 16S gene is separated from the 23S-5S genes (Chen and Finch 1989); in addition, a truncated third copy (pseudogene) of 16S is present in one strain (Skamrov et al. 1995). *M. fermentans* also has two sets of rRNA genes, but the 16S-23S clusters are located closely together in a tail-to-tail fashion while both 5S genes are widely separated (Huang et al. 1995).

Relatively little is known about transcriptional regulation in mycoplasmas. It has been suggested that rRNA synthesis in *M. capricolum* is linked with ppGpp and pppGpp accumulation in cells (Glaser et al. 1981) in a manner analogous to the negative stringent contol seen in *E. coli* (Condon et al. 1995), although it appears that the control mechanism involving ppGpp in *M. capricolum* is different from that of *E. coli* (Gafny et al. 1988).

However, the parasitic lifestyle of mycoplasmas ensures a fairly homogenous environment which may preclude the need for any sort of variable control of rRNA expression (Muto et al. 1992). The normal 16S-23S-5S arrangement of rRNA genes ensures that all three molecules are present in equimolar amounts in the cell (Condon et al. 1995). In order to preserve the stoichiometry necessary for complete ribosomes in *M. hyopneumoniae* and *M. flocculare*, therefore, the transcriptional control of both operons must be tightly regulated. However, the regions upstream of the *M. hyopneumoniae* and *M. flocculare* 5S genes show considerable heterology between the two species (Stemke et al. 1994a). Two TATAAT Pribnow box sequences were seen at positions -10 and -25 in *M. flocculare*, whereas only the -10 sequence was seen in *M. hyopneumoniae*, preceded by a long poly-A tract (>40) which was not present in *M. flocculare*. It is possible that only the one (homologous) Pribnow box acts as a promoter in *M. flocculare*, although no transcriptional analysis of these operons has been done. Neither 5S upstream region is very similar to to the 16S-23S promoter, suggesting the operons may be controlled in different manners.

The exceptionally slow growth rates of both species in culture (Kobisch and Friis 1996) could be related to an inability to properly synthesize ribosomes, as might occur if the three rRNA species are not present in equal amounts. Because the 16S-23S operon is quite near to the origin of replication (Figure 3-17), two copies of it are capable of being transcribed during replication until the replication fork reaches the more distant 5S gene. This could represent a wasteful accumulation of the larger rRNA molecules, potentially causing strain on the cell's energy resources without productive ribosome formation. Whether or not the two promoters for the 16S-23S operon may help modulate this effect, or if the 5S operon is more active to compensate for this, or if such a dosage effect would even have a substantial effect in genomes of this size remains to be determined.

A previous report had indicated that the next nearest characterized relative of *M. hyopneumoniae* and *M. flocculare*, *M. hyorhinis* (Stemke et al. 1992) carries only a single, conventional 16S-23S-5S rRNA operon (Göbel et al. 1984). However, that study had used a single 23S-5S probe 2.6 kb in length to locate those particular genes. Work done in this laboratory (Y. Huang, Ph.D. thesis, University of Alberta, Edmonton AB, 1994) showed that using separate probes for each rRNA gene, the 5S probe never hybridized to the same restriction fragment as the 23S probe. This strongly suggested that *M. hyorhinis* shares a similar type of rRNA gene arrangement and that the genomic events leading to it occurred in an ancestor of this line. The conservation of neighboring genes (namely *glyT*, *ptsII*, and *recA*) within approximately 100 kb of the 5S gene in both *M. hyopneumoniae* and *M. flocculare* (fragments 1.4 and 9.4, respectively; see Figures 3-8 and 3-17) strongly suggests that the event occurred only once.

Two possible events may have occurred: separation of the 5S gene from a single operon (either by transposition or inversion) to a separate location; or the initial presence of two rRNA operons, followed by subsequent deletion of the 5S gene from one copy and the 16S-23S genes from the other. The single operon hypothesis would require that the 5S gene be placed in front of a suitable promoter immediately and the missing terminator be replaced downstream of the 23S gene, seemingly an unlikely possibility until it is considered that this may have occurred not once but twice in *M. fermentans* (Huang et al. 1995). However, given the propensity of mycoplasmas to discard redundant genetic information which does not directly enhance their chances of parasitic survival (e.g. genes involved antigenic variation, etc.), the two operon hypothesis is also attractive. It is possible that *M. fermentans* had undergone duplication of part of its genome after such an event. In order to determine which of these alternatives is correct, it may be useful to compare promoter and terminator sequences from these porcine mycoplasmas to those from their closest relatives with both one and two conventional rRNA operons, as well as examine the surrounding gene organization. However, given the genomic plasticity of mycoplasmas and their high mutation rate, any useful information of this sort may already be lost in evolutionary "noise".

4.5.2 Insertion sequence IS1221

DNA hybridization studies between porcine mycoplasmas (Taylor et al. 1988) had identified a highly repeated sequence from *M. hyorhinis* which hybridized to a single 8.0 kb *Eco*RI fragment from *M. hyopneumoniae* strain J as well as a 5.9 kb *Eco*RI fragment from *M. flocculare*. Sequence analysis (Ferrell et al. 1989) identified it as an 1.5 kb insertion sequence-like element (IS1221) of which two copies were actually present in the *M. hyopneumoniae* 8.0 kb fragment. Southern blotting experiments showed that at least 2-3 copies of it were also present in several field isolates of *M. hyopneumoniae* but that it was not present at all in *M. hyopneumoniae* strain VPP11.

In this study, plasmid clone 25-1a, which contains a portion of the IS1221 sequence, hybridized to a single location in both of the genomes examined, although it could not be determined whether IS1221 is also tandemly repeated in *M. flocculare*. The putative transposase genes in both copies of IS1221 in *M. hyopneumoniae* strain J are assumed to be inactive due to mutation (Zheng and McIntosh 1995) and it is quite likely that the same is true for *M. flocculare*, since its copy number is limited in this species as well. The fact that *M. hyorhinis*, *M. hyopneumoniae*, and *M. flocculare* all share the common environment of the porcine respiratory tract presents the possibility that separate interspecies transfer events of this mobile genetic element from *M. hyorhinis* to both *M. hyopneumoniae* and *M. flocculare* have occurred. However, in both *M. hyopneumoniae* and *M. flocculare* IS1221 is closely associated with the DNA sequences cloned in plasmids B5-1b and B7-1b and is located within approximately 100 kb of the *samS* gene (Figures 3-8 and 3-17). Since all three of these porcine mycoplasmas are closely related (Stemke et al. 1992), it is possible that IS1221 was present in the precursor species of *M. hyopneumoniae* and *M. flocculare*, and is perhaps even a genetic remnant of their common ancestry with *M. hyorhinis*.

If multiple horizontal DNA transmission events have taken place, the close association with B5-1b and B7-1b could indicate a preferred site of integration for IS1221. Alternatively, these sequences may be part of a larger mobile genetic element also including IS1221. It would be telling to determine whether B5-1b and B7-1b are associated with any of the copies of IS1221 in *M. hyorhinis* and other strains of *M. hyopneumoniae*, as well as the relative chromosomal location of *samS*.

4.5.3 Chromosomal rearrangements

Placement of genetic markers on the physical maps of both *M. hyopneumoniae* and *M. flocculare* can help identify the types of chromosomal rearrangements that have occurred since the two species have diverged from their common ancestor and may explain the differences in pathogenicity between them. Twenty-one of the markers placed on the *M. hyopneumoniae* chromosome were successfully located on that of *M. flocculare* giving a good basis for comparison of the two genomes. Unfortunately, the lower restriction site resolution of the *M. flocculare* map does not permit the precise ordering of genes in some cases, and very often precise distances between genes cannot be determined. However, several striking differences in gene arrangements between the two species can be noted, and these may be explained by as few genomic rearrangements as three chromosomal inversions. It is entirely possible other chromosmal events have gone undetected due to the low resolution of the maps, particularly that of *M. flocculare*.

One relatively small inversion, illustrated in Figure 4-2, involved the chromosomal origin of replication region (*oriC*, as indicated by the *dnaA* gene) and the 16S-23S rRNA operon, and was flanked by the nearby *rpoB* and *valS* genes. This is the most parsimonious event that could have occurred in this region, for the precise order of *dnaA* and *valS* in *M. hyopneumoniae*, and of *dnaA* and *rpoB* in *M. flocculare* is not known. Further inversions may have occurred if finer resolution mapping reveals the *dnaA* gene to be outside either one of these two markers. It is important to note that an inversion involving the origin of replication itself would preserve the orientation of genes relative to the direction of replication, thereby minimizing interference between DNA and RNA polymerases.

It was previously thought that eubacterial *oriC* regions were extremely well conserved in terms of gene content and order. Comparison of the *dnaA* regions from *E. coli*, *Bacillus subtilis*, *Pseudomonas putida*, and *Micrococcus luteus* – a rather diverse selection of bacteria – revealed a very conserved gene order of *rnpA-rpmH-dnaA-dnaN-recF-gyrB*, with an untranslatable gap between *rpmH* and *dnaA* containing several DnaA boxes and thought to be the location of strand separation where DNA polymerase activity begins (Ogasawara et al. 1990). All genes in this vicinity are transcribed in the direction away from this gap. As well, *gyrA* is generally linked to the *gyrB* gene in Gram-positive bacteria (Margerrison et al. 1992; Ye et al. 1994b), to which the mycoplasmas are phylogenetically related (Weisburg et al. 1989). However, none of the mollicutes thus far studied share this conserved order. The *Spiroplasma citri oriC* most closely resembles it, although the *recF* gene is missing (Ye et al. 1994b). In fact, *recF* is not present in any of

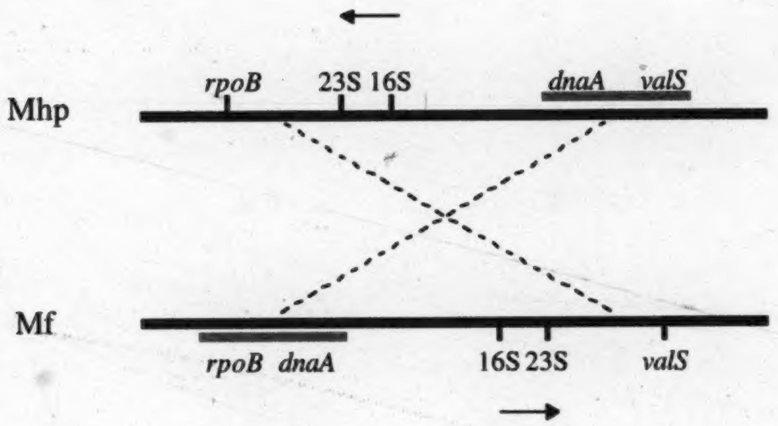
Mhp
rpoB
23S
16S
dnaA
valS
Mf
rpoB
dnaA
16S
23S
valS

Figure 4-2. Schematic diagram of crossover event leading to chromosomal inversion in the *oriC* region of *M. hyopneumoniae* (Mhp) and *M. flocculare* (Mf). Arrows indicate direction of transcription of rRNA operon. Grey bars indicate ambiguity in actual order of genes. Gene symbols are as in Tables 3-1 and 3-2.

the characterized mollicutes *dnaA* regions and has not been found in either of the fully sequenced mycoplasma genomes (Fraser et al. 1995; Himmelreich et al. 1996). In both *M. genitalium* and *M. pneumoniae* the *dnaA* and *dnaN* genes are located on opposite sides of the untranslatable gap, and the only difference between these two closely related bacteria in this region is the presence of an unidentified glutamine and proline rich protein in *M. pneumoniae* (Himmelreich et al. 1997). The genes for DNA gyrases A and B are separated by about 35 kb in *M. hominis* (Ladefoged and Christiansen 1994), although the relative location of *dnaA* is not known. Finally, two dimensional gel-electrophoretic analysis showed that replication initiation occurred in a conventional rnpA-rpmH-dnaA-dnaN region in *M. capricolum* (Miyata et al. 1993), but its *gyrB* and *gyrA* genes (which are linked) are located opposite from *oriC* on the circular chromosome (Sano and Miyata 1994).

These examples all indicate how the remarkably high level of genomic plasticity in mollicutes is not even constrained by the biological significance and degree of conservation of the *oriC* region. The inversion of *dnaA* and the rRNA genes between *M. hyopneumoniae* and *M. flocculare* demonstrates that this is also true in these species, as does the location of *gyrA* approximately 160 kb (intervening fragments 3.6b, 3.9a, 3.10, and 3.5) away from *dnaA* in *M. hyopneumoniae* (Figure 3-17).

Two overlapping large inversions can also be deduced by comparing the two genetic maps. Figure 4-3 illustrates the genomic organization of a hypothetical ancestor or intermediate of *M. hyopneumoniae* and *M. flocculare*, and indicates the inversions which may have occurred since the two species diverged. One involves a region of up to 245 kb in *M. flocculare* (contained in fragments 7.4, 7.7, and 7.6) and included the genes mapping near the 5S rRNA gene as well as *nrdF* and *ldh*, which encode major antigens in *M.*

hyopneumoniae. The second inversion covers up to 370 kb of the *M. hyopneumoniae* genome (fragments 2.2, 2.7a, 2.3, and 2.8) and again involves *oriC* as well as some of the genes involved in the previously mentioned inversion. It cannot be determined from this study which of the inversions occurred first, or if both had occurred since divergence, but genomic analysis of a related organism such as *M. hyorhinis* may provide more clues.

The gene for the *M. hyopneumoniae* ciliary adhesin (*adh*) was not detected in the *M. flocculare* chromosome, and it has previously been shown that it is not present in *M. flocculare* or *M. hyorhinis* (Wilton et al. 1998). In *M. hyopneumoniae* this gene is associated with several of the genes that were part of one of the inversions, and could possibly have been lost during the chromosomal rearrangement. Depending on the actual distance of *pyk* and *ldh* from *nrdF* and *hsp60* – up to approximately 150 kb (3.2c and 3.8a) in *M. hyopneumoniae*, but less than 75 kb (overlap between 7.6 and 9.5c) in *M. flocculare* – this could represent one of the major determinants of the differences between *M. hyopneumoniae* and *M. flocculare*, in terms of both genome size and pathogenicity. Characterization of particular cosmids (namely 32.1, 543, and 43) will help to better determine the genomic organization of this region and possibly identify other genes which are not present in *M. flocculare*, provided that *adh*, *pyk*, and *ldh* are not in the nearby uncloned region. Although no other obvious large regions of deletion in *M. flocculare* could be observed in this study, attrition of other as yet uncharacterized individual genes could further explain the differences in pathogenicity between the two species.

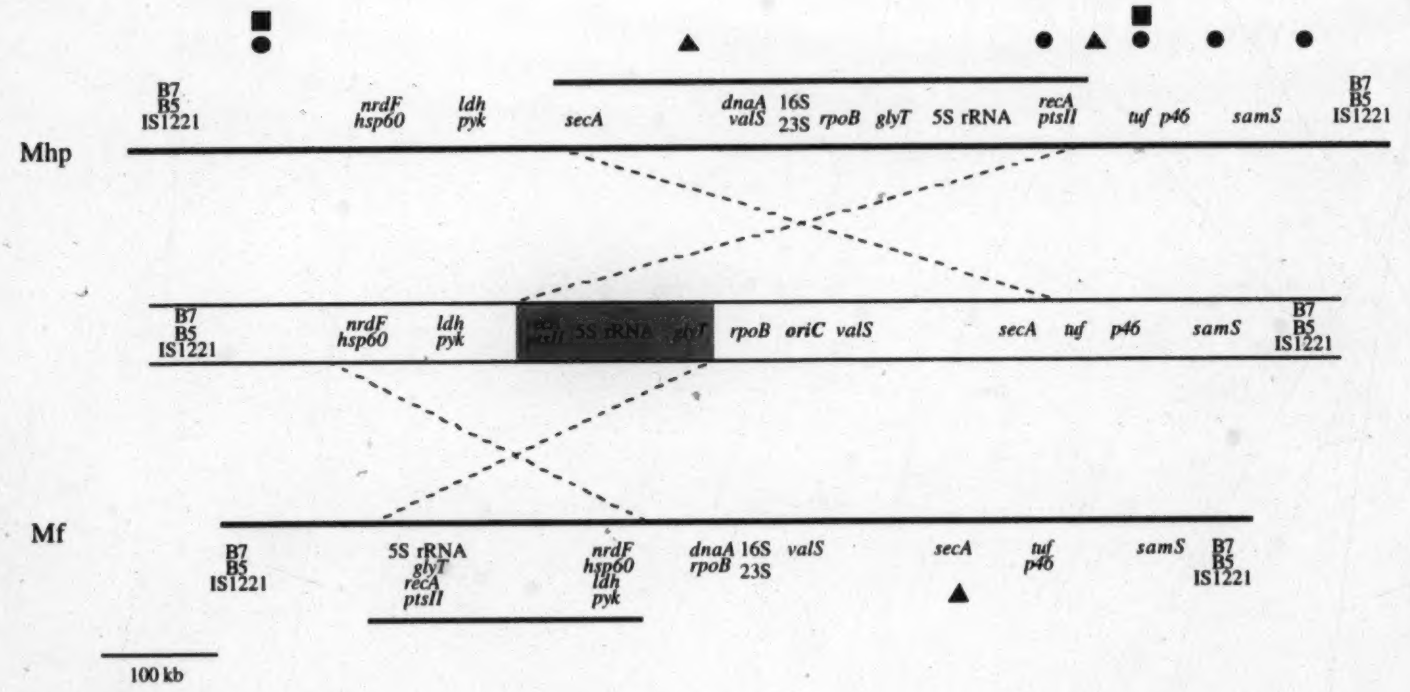
Mhp
B7 B5 IS1221
nrdF hsp60
ldh pyk
secA
dnaA valS
16S 23S
rpoB
glyT
5S rRNA
recA ptsII
tuf
p46
samS
B7 B5 IS1221
B7 B5 IS1221
nrdF hsp60
ldh pyk
recA ptsII
5S rRNA
glyT
rpoB
oriC
valS
secA
tuf
p46
samS
B7 B5 IS1221
Mf
B7 B5 IS1221
5S rRNA glyT recA ptsII
nrdF hsp60 ldh pyk
dnaA rpoB
16S 23S
valS
secA
tuf p46
samS
B7 B5 IS1221
100 kb

Figure 4-3. Schematic diagram of large chromosomal inversions in *M. hyopneumoniae* (Mhp) and *M. flocculare* (Mf) with hypothetical ancestor or intermediate chromosome. Maps were linearized at the conserved region containing the IS1221 element. 16S-23S rRNA and *dnaA* genes are indicated as *oriC* in hypothetical chromosome since gene order is not known. Grey bars indicate inverted regions and shaded area has been involved in two inversions. *M. hyopneumoniae* repetitive elements *repJ* (●), B28-1b (■), and 13-1a (▲) are also indicated.

Chromosomal rearrangements are generally the result of homologous recombination between repeated sequences within the genome (Mahan et al. 1990). The genomes of *M. pneumoniae* and *M. genitalium* can be divided into six segments in which gene organization is very highly conserved, although these segments are differently arranged (Himmelreich et al. 1997). These rearrangements are believed to have been mediated by homologous recombination between repetitive DNA sequences flanking each segment. The presence of the gene for the site-specific recombinase RecA in both *M. hyopneumoniae* and *M. flocculare* indicates that homologous recombination is possible in these organisms – it is believed that some mollicutes may lack this activity (Marais et al. 1996). The two 13-1a elements in *M. hyopneumoniae* are located close to the ends of one of the inversions (Figure 4-3), although one is inside of the region, with *secA* defining the leftmost boundary. Furthermore, only one copy of 13-1a is present in *M. flocculare*. It is possible that if recombination between the 13-1a elements occurred, a more recent, smaller inversion switched the positions of *secA* and 13-1a in *M. hyopneumoniae* and the second copy was deleted in *M. flocculare*. Attempts to detect *repJ* and 28-1b in the *M. flocculare* genome were not successful, although whether this is due to absence of the genes or experimental error is not known. However, the order of genes mapping near to these elements appears to remain conserved in *M. flocculare* (once the two large inversions are accounted for), suggesting they have not been involved in recombination activity since the two species diverged. Illegitimate recombination has been suggested as the cause of plasmid deletions in mollicutes (Dybvig 1989; King and Dybvig 1993) and could possibly be involved in chromosomal deletion and rearrangement, although the plasmids in those studies replicate via a rolling circle mechanism with a single-stranded intermediate which may be particularly prone to such events.

Several likely functional barriers to the formation of inversions in bacterial chromosomes have been described (Mahan et al. 1990), including the rearrangement of sites that could disrupt chromosomal replication, particularly involving disruption of the terminus region and orientation of highly transcribed genes with regard to the direction of replication fork movement. The genomic region containing IS1221 managed to remain approximately half a genome length away from *dnaA* (~500 kb in *M. hyopneumoniae*, 450 kb in *M. flocculare*) throughout the course of the described inversions. Specific mechanisms exist for the termination of chromosomal replication in *E. coli* and *B. subtilis* (Kuempel et al. 1990; Wake 1990) involving particular sequences and regions of their respective chromosomes. No such features have been specifically described in the sequenced mycoplasma genomes (Fraser et al. 1995; Himmelreich et al. 1996), although the preservation of the IS1221 region opposite from *oriC* in both *M. hyopneumoniae* and *M. flocculare* could suggest some functional significance for this region. As for the interruption of the replication fork by RNA polymerases, a region including at least two genes that are presumably highly transcribed (5S rRNA and Gly-tRNA) does appear to have undergone an inversion, implying that at least in these mycoplasmas, gene orientation is not a major concern. However, transcription away from the origin of replication is maintained for the 16S-23S operon.

Too little is known about mycoplasmal gene expression coordination and chromosomal tertiary structure to comment on other possible barriers to inversion, such as gene dosage effects and rearrangement of sites that determine the condensed structure of the chromosome (Mahan et al. 1990). However, the small size and rather basic nature of mycoplasma genomes may preclude such effects.

4.6 Errors in sizing of DNA fragments by PFGE

Although the genome sizes most of the genetic distances thus far reported in this study are based on the measurement of macrorestriction fragments by CHEF, certain anomalies in the data indicate discrepancies between DNA fragment lengths determined by PFGE and conventional gel electrophoresis. The best characterized of these involves *Apa*I fragment 1.7, which was measured as approximately 45 kb by CHEF. However, transcript probes from either end of cosmid insert 129 (determined by *Eco*RI restriction analysis to be 36.5 kb in length) hybridize with fragments 1.10 and 1.12, which flank fragment 1.7b. Furthermore, conventional gel electrophoresis on a 0.4% agarose gel of *Apa*I-digested cosmid 129 yields two fragments of approximately 14 kb and 28 kb (not shown). The shorter fragment presumably corresponds with the 5.2 kb pcosRW2 vector (which does not contain an *Apa*I site) and the cloned portions of fragments 1.10 and 1.12, leaving the 28 kb fragment to represent 1.7b.

The possibility that a portion of the cosmid 129 insert was deleted by the *E. coli* host was ruled out by the fact that contig II is quite well characterized by virtue of the considerable number of overlapping cosmids found in that region (Figures 3-13 and 3-14). The likelihood that all of the cosmids overlapping with 129 had undergone an identical deletion is extremely small. There is a possibility that the *M. hyopneumoniae* culture used to construct the cosmid library had undergone a deletion in this region, but such an event should only have resulted in a subpopulation of deleted DNA which would presumably be underrepresented in the library. Similar size discrepancies are also seen in other parts of the genome. For instance, the *Eco*RI fragments of cosmids 214, 1, and 45 total 71.5 kb, yet hybridization data shows that *Asp*718 fragments 3.6b, 3.9a, and 3.10 are completely contained within this subcontig, even though CHEF analysis puts the sum of their sizes at approximately 80 kb – not including the overlap of cosmid 214 with 3.2b and 45 with 3.5.

It has been suggested (Maniloff 1989) that the low G+C content of mycoplasma genomes leads to slower than expected mobilities of DNA fragments during PFGE and that genome sizes derived in this manner are overestimated. This notion was put forward to support the theory that mycoplasma genome sizes fall into two distinct size categories, clustering around 760 kb and 1600 kb, as determined by methods such as renaturation kinetics and electron microscopy. Comparison of G+C content with apparent genome sizes as determined by PFGE for five *Mycoplasma* species, supposedly of the "small" genome variety, showed a linear relationship, with the more A+T rich genomes appearing larger (Maniloff 1989). Later studies sampling a larger number of mollicutes genomes (Neimark and Lange 1990; Robertson et al. 1990) have dispelled the idea of a correlation between base composition and PFGE genome sizes, but this does not specifically address the effect of G+C content on PFGE.

There have been few experimental data published on the effect of base composition on DNA mobility during PFGE. The apparent sizes of concatamers of DNA from the temperate phages RP2 and RP3 (64.7 and 62.4 kb, respectively; 70% G+C) of *Streptomyces rimosus* were compared with λ ladders (48.5 kb; 50% G+C) after CHEF electrophoresis (Gravius et al. 1994). It was found that the G+C rich fragments (particularly the lower molecular weight concatamers) migrated relatively faster than the λ molecules. The relative rate of error followed a linear regression and ranged from 7.5% for a 500 kb fragment to over 30% for a 60 kb fragment. If the converse is true for A+T rich DNA, the possibility exists that some mollicutes genome sizes have been overestimated, since λ ladders and yeast chromosomes have been the only molecular weight markers used for the determination of mycoplasma genome sizes by PFGE.

How base composition can affect migration of DNA during PFGE is not known, because the actual physical basis for PFGE is not completely understood. Fluoresence microscopy of individual DNA molecules (Gurrieri et al. 1990) showed that when a molecule aligned by an electrical field (which has then been switched off) has another electrical field applied to it in a different direction, a number of kinks form in the direction of the second field. These kinks grow in length with time and compete with one another until one becomes the new "head" of the molecule which leads its migration through the gel. The amount of time each molecule takes to finally orient itself in this mannner is dependant upon length, thereby leading to separation of differently sized molecules. What causes these kinks in the DNA to form, and what determines which of these becomes the new head is not known; base composition could potentially affect this process in several ways.

It is believed that base composition has an effect on the flexibility of DNA molecules (Hagerman 1988), although experimental evidence is somewhat ambiguous. If A+T rich sequences are indeed less flexible, kink formation may be impeded, resulting in longer reorientation times for such molecules. A+T rich tracts have also been implicated in causing reduced mobility of DNA during conventional electrophoresis through polyacrylamide gels – although not agarose gels – due to effects on the intrinsic curvature of the molecules (Hagerman 1986). Another possibility is that A+T rich sequences are more susceptible to localized melting of the DNA strands (Cantor et al. 1988) due to weaker hydrogen bonding when placed under stress during kink formation. Such partial denaturation would cause a marked reduction in mobility; a similar principle is utilized in denaturing gradient gel electrophoresis (Poddar and Maniloff 1989). This may also partly explain the sensitivity of PFGE separations to minor changes in running temperature

(Cantor et al. 1988). This theory could be easily tested: if true, low G+C content mycoplasma DNA would run relatively slower compared to λ concatamers at 20 °C than at 13 °C, although overall migration speed would increase with temperature.

The genome size of *M. genitalium* as determined by PFGE (~600 kb) (Colman et al. 1990) compares favorably with its complete genome sequence (580 kb) (Fraser et al. 1995). However, comparison of the genome size of *M. capricolum* (24% G+C) as determined by PFGE (Whitley et al. 1991) with that determined by two-dimensional denaturing gradient gel electrophoresis (Poddar and Maniloff 1989) gives a very large discrepancy (1070 kb vs. 724 kb, respectively) which cannot be readily explained. In the same study, the latter method produced values for the genome sizes of *Acholeplasma laidlawii* (32% G+C) and *Haemophilus influenzae* (38% G+C) which agree closely with PFGE analysis and, in the case of *H. influenzae*, the complete genome sequence (Fleischmann et al. 1995; Lee and Smith 1988; Neimark and Lange 1990; Robertson et al. 1990).

Analysis of 214 kb of sequence data from *M. capricolum* (Bork et al. 1995) indicates only 64% of the genome encodes functional genes, compared to a coding density of 88% in *M. genitalium* (Fraser et al. 1995). It is possible that total genomic G+C content is a relative indicator of coding density, since functionally less important regions would have a lower G+C content as a reflection of the mutational bias in mycoplasmas and the lack of sequence constraints in those regions (Muto 1987). Base composition variation throughout individual mycoplasma genomes has been noted on more than one occasion (Cocks et al. 1989; Himmelreich et al. 1997; Kerr et al. 1997; Pyle and Finch 1988), although its biological significance is unclear. In practical terms, however, it may have consequences on experimental data. Depending on the method used and degree of fragmentation of chromosomal DNA, G+C content determination can be inconclusive; witness the discrepancy in *M. hyopneumoniae* values between Stemke et al (1985) (33%) and Kirchhoff and Flossdorf (1987) (27.5%). More to the point of this study, the possibility exists that longer A+T rich tracts may have a disproportionate effect on PFGE-determined genome sizing and as a result, certain genomic fragments may run more closely to their "true" sizes than others. For instance, the overlap between fragments 1.3, 2.1, 3.2c, and 10.2 (Figure 3-15) is measured at approximately 100 kb without a recognition site for any of the four restriction enzymes used in this study. This indicates that this region may be particularly A+T rich, thereby casting doubt on its actual size. Coincidentally, this region corresponds to one of the gaps in the overlapping cosmid library, implying that DNA sequences from this region are unstable in *E. coli*.

It may be helpful to consider the base composition of the molecular weight markers used when performing PFGE analysis of mollicutes genomes. A 16 kb DNA virus from *Spiroplasma citri* has been described (Maniloff 1992a) which presumably has a G+C content similar to its host (25-27%). It possesses cohesive ends which would make it amenable to concatamerization and use as a lower molecular weight marker. Ultimately, however, until the limitations of PFGE are understood and quantified (particularly with respect to variation of base composition within a genome and A+T rich tracts), the only precise method of determining the size of a mycoplasma genome is complete sequencing (Fraser et al. 1995; Himmelreich et al. 1996) or at the least, construction of a complete overlapping clone library (Lucier et al. 1994; Wenzel et al. 1992)..

4.7 Summary

To conclude this report, the results of this study can be summarized as follows. *M. hyosynoviae* and *M. hyopharyngis*, two mycoplasma species of isolated from pigs were shown by 16S rRNA phylogeny not to be specifically related to each other nor to a previously described cluster of related porcine mycoplasmas, *M. hyorhinis*, *M. hyopneumoniae*, and *M. flocculare*. Rather, the closest characterized relatives of each are *M. orale* and *M. salivarium* (for *M. hyosynoviae*) and *M. lipophilum* (for *M. hyopharyngis*), all of which are part of the normal human oral microflora. Low resolution restriction maps of the genomes of *M. hyopneumoniae* (a respiratory pathogen) and *M. flocculare* (a commensal organism) were created by the use of CHEF gel electrophoresis and Southern hybridization. Placement of genetic markers on these maps allowed the identification of three genomic regions which have been involved in inversions since these two species diverged, as well as identifying a genomic region (near the *adh* gene in *M. hyopneumoniae*) which may help account for the differences in pathogenicity between the two. An overlapping cosmid library covering most of the *M. hyopneumoniae* genome was also constructed, which could facilitate closer study of this region and others of interest, in hopes of identifying as yet uncharacterized genes which may encode suitable therapeutic or vaccination targets to help control *M. hyopneumoniae* pneumonia.

5. REFERENCES

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990). "Basic local alignment search tool." *J. Mol. Biol.*, **215**: 403-410.

Amikam, D., Glaser, G., and Razin, S. (1984). "Mycoplasmas (*Mollicutes*) have a low number of rRNA genes." *J. Bacteriol.*, **158**: 376-378.

Anagnostopoulos, C., Piggot, P. J., and Hoch, J. A. (1993). "The genetic map of *Bacillus subtilis*." In *Bacillus subtilis and other Gram-positive bacteria*, A. L. Sonenshein, J. A. Hoch, and R. Losick, eds., American Society for Microbiology, Washington DC, 425-461.

Armstrong, C. H., Sands-Freeman, L., and Freeman, M. J. (1987). "Serological, pathological and cultural evaluations of swine infected experimentally with *Mycoplasma flocculare*." *Can. J. Vet. Res.*, **51**: 185-188.

Artiushin, S., and Minion, F. C. (1996). "Arbitrarily primed PCR analysis of *Mycoplasma hyopneumoniae* field isolates demonstrates genetic heterogeneity." *Int. J. Syst. Bacteriol.*, **46**: 324-328.

Asai, T., Okada, M., Ono, M., Irisawa, T., Mori, Y., Yokomizo, Y., and Sato, S. (1993). "Increased levels of tumor necrosis factor and interleukin-1 in bronchoalveolar lavage fluid from pigs infected with *Mycoplasma hyopneumoniae*." *Vet. Immunol. Immunopathol.*, **38**: 253-260.

Asai, T., Zaporojets, D., Squires, C., and Squires, C. L. (1999). "An *Escherichia coli* strain with all chromosomal rRNA operons inactivated: complete exchange of rRNA genes between bacteria." *Proc. Natl. Acad. Sci.*, **96**: 1971-1976.

Bachmann, B. (1990). "Linkage map of *Escherichia coli* K-12, edition 8." *Microbiol. Rev.*, **54**: 130-197.

Baril, C., Richaud, C., Baranton, G., and Girons, I. S. (1989). "Linear chromosome of *Borrelia burgdorferi*." *Res. Microbiol.*, **140**: 507-516.

Barile, M. F. (1983). "Arginine hydrolysis." In *Methods in Mycoplasmology*, S. Razin and J. G. Tully, eds., Academic Press, New York, 345-349.

Bautsch, W. (1988). "Rapid physical mapping of the *Mycoplasma mobile* genome by two-dimensional field inversion gel electrophoresis techniques." *Nucleic Acids Res.*, **16**: 11461-11467.

Bautsch, W. (1994). "Bacterial genome mapping by two-dimensional pulsed-field gel electrophoresis (2D-PFGE)." *Mol. Biotech.*, **2**: 29-44.

Berner, H. (1995). "Impfung -- eine neue Methode der Bekämpfung der Enzootischen Pneumonie des Schweines." *Der praktische Tierarzt*, **8:**: 668-682.

Bhugra, B., and Dybvig, K. (1993). "Identification and characterization if IS*1138*, a transposable element from *Mycoplasma pulmonis* that belongs to the IS*3* family." *Mol. Microbiol.*, **7**: 577-584.

Bhugra, B., Voelker, L. L., Zou, N., Yu, H., and Dybvig, K. (1995). "Mechanism of antigenic variation in *Mycoplasma pulmonis*: interwoven, site-specific DNA inversions." *Mol. Microbiol.*, **7**: 577-584.

Birren, B., and Lai, E. (1993). *Pulsed Field Gel Electrophoresis: A Practical Guide*, Academic Press, San Diego CA.

Boatman, E. S. (1973). "Morphology and ultrastructure of mycoplasmas." *Ann. N.Y. Acad. Sci*, **225**: 63-102.

Boatman, E. S. (1979). "Morphology and ultrastructure of mycoplasmas." In *The Mycoplasmas I: Cell Biology*, M. F. Barile and S. Razin, eds., Academic Press, New York, 63-102.

Bölske, G., Strandberg, M.-L., Bergström, K., and Johansson, K.-E. (1987). "Species-specific antigens of *Mycoplasma hyopneumoniae* and cross-reactions with other porcine mycoplasmas." *Curr. Microbiol.*, **15**: 233-239.

Bork, P., Ousounis, C., Casari, G., Schneider, R., Sander, C., Dolan, M., and others. (1995). "Exploring the *Mycoplasma capricolum* genome: a minimal cell reveals its physiology." *Mol. Microbiol.*, **16**: 955-967.

Bredt, W. (1973). "Motility of mycoplasmas." *Ann NY Acad Sci*, **225**: 246-250.

Brosch, R., Gordon, S. V., Billault, A., Garnier, T., Eiglmeier, K., Soravito, C., and others. (1998). "Use of a *Mycobacterium tuberculosis* H37Rv bacterial artificial chromosome library for genome mapping, sequencing, and comparative genomics." *Infect. Imm.*, **66**: 2221-2229.

Buttenschøn, J., Friis, N. F., Aalbæk, B., Jensen, T. K., Iburg, T., and Mousing, J. (1997). "Microbiology and pathology of fibrinous pericarditis in Danish slaughter pigs." *J. Vet. Med.*, **44**: 271-280.

Cantor, C. R., Smith, C. L., and Mathew, M. K. (1988). "Pulsed-field gel electrophoresis of very large DNA molecules." *Ann. Rev. Biophys. Biophys. Chem.*, **17**: 287-304.

Carle, G. F., Frank, M., and Olson, M. V. (1986). "Electrophoretic separations of large molecules by periodic inversion of the electric field." *Science*, **232**: 65-68.

Carle, G. F., and Olson, M. V. (1984). "Separation of chromosomal DNA molecules from yeast by orthagonal-field-alteration gel electrophoresis." *Nucleic Acids Res.*, **12**: 5647-5664.

Chan, H. W., and Ross, R. F. (1984). "Restriction endonuclease analyses of two porcine mycoplasma deoxyribonucleic acids: species-specific methylation in the *Mycoplasma hyopneumoniae* genome." *Int. J. Syst. Bacteriol.*, **34**: 16-20.

Chen, J.-R., Lin, J.-H., Weng, C.-N., and Lai, S.-S. (1998). "Identification of a novel adhesin-like glycoprotein from *Mycoplasma hyopneumoniae*." *Vet. Microbiol.*, **62**: 97-110.

Chen, X., and Finch, L. R. (1989). "Novel arrangement of rRNA genes in *Mycoplasma gallisepticum*: separation of the 16S gene of one set from the 23S and 5S genes." *J. Bacteriol.*, **171**: 2876-2878.

Chomczynski, P. (1992). "One-hour downward alkaline capillary transfer of DNA and RNA." *Anal. Biochem.*, **201**: 134-139.

Chou, S.-Y., Chung, T.-L., Chen, R.-J., Ro, L.-H., Tsui, P.-I., and Shiuan, D. (1997). "Molecular cloning and analysis of a HSP (heat shock protein)-like 42 kDa antigen gene of *Mycoplasma hyopneumoniae*." *Biochem. Mol. Biol. Int.*, **41**: 821-831.

Chu, G., Vollrath, D., and Davis, R. W. (1986). "Separation of large DNA molecules by contour-clamped homogenous electrical fields." *Science*, **234**: 1582-1585.

Ciprian, A., Pijoan, C., Cruz, T., Camacho, J., Tortora, J., Colmanares, G., and others. (1988). "*Mycoplasma hyopneumoniae* increases the susceptibility of pigs to experimental *Pasteurella multocida* pneumonia." *Can. J. Vet. Res.*, **523**: 434-438.

Clyde, W. A., and Hu, P. C. (1986). "Antigenic determinants of the attachment protein of *Mycoplasma pneumoniae* shared by other pathogenic *Mycoplasma* species." *Infect. Immun.*, **51**: 690-692.

Cocks, B. G., Pyle, L. E., and Finch, L. R. (1989). "A physical map of the genome of *Ureaplasma urealyticum* 960T with ribosomal RNA loci." *Nucleic Acids Res.*, **17**: 6713-6719.

Cole, B. C. (1996). "Mycoplasma interactions with the immune system: implications for disease pathology." *ASM News*, **62**: 471-475.

Cole, B. C., Knudtson, K. L., Oliphant, A., Sawitzke, A. D., Pole, A., Manohar, M., and others. (1996). "The sequence of the *Mycoplasma arthritidis* superantigen MAM: identification of functional domains and comparison with microbial superantigens and plant lectin mitogens." *J. Exp. Med.*, **183**: 1105-1110.

Cole, B. C., and Ward, J. R. (1979). "Mycoplasmas as arthritogenic agents." In *The Mycoplasmas Vol. 2: Human and Animal Mycoplasmas*, J. G. Tully and R. F. Whitcomb, eds., Academic Press, New York, 367-398.

Cole, R. M., Tully, J. G., Popkin, T. J., and Bové, J. M. (1973). "Morphology, ultrastructure, and bacteriophage infection of the helical mycoplasma-like organisms (*Spiroplasma citri* gen. nov., sp. nov.) cultured from "stubborn" disease of citrus." *J. Bacteriol.*, **115**: 367-386.

Colman, S. D., Hu, P.-C., Litaker, W., and Bott, K. F. (1990). "A physical map of the Mycoplasma genitalium genome." *Mol Microbiol*, **4**: 683-687.

Condon, C., Squires, C., and Squires, C. L. (1995). "Control of rRNA transcription in *Escherichia coli*." *Microbiol. Rev.*, **59**: 623-645.

Cordwell, S., Basseal, D. J., Pollack, J. D., and Humphrey-Smith, I. (1997). "Malate/lactate dehydrogenase in Mollicutes: evidence for a multienzyme protein." *Gene*, **195**: 113-120.

Cote, R. (1992). *Catalogue of Bacteria and Phages*, American Type Culture Collection, Rockville, MD.

Crespi, M., Messens, E., Caplan, A.-B., Monagu, M. v., and Desomer, J. (1992). "Fasciation induction by the phytopathogen *Rhodococcus fascians* depends upon a linear plasmid encoding a cytokinin synthase gene." *EMBO J.*, **11**: 795-804.

Dienes, L., and Weinberger, H. J. (1951). "The L-forms of bacteria." *Bact. Rev.*, **15**: 245-288.

DiLella, A. G., and Woo, S. L. C. (1987). "Cloning large segments of genomic DNA using cosmid vectors." *Met. Enz.*, **152**: 199-212.

Dohoo, I. R., and Montgomery, M. E. (1996). "A field trial to evaluate a *Mycoplasma hyopneumoniae* vaccine: effects on lung lesions and growth rates in swine." *Can. Vet. J.*, **37**: 299-302.

Dybvig, K. (1989). "Transformation of *Acholeplasma laidlawii* with streptococcal plasmids pVA868 and pVA920." *Plasmid*, **21**: 155-160.

Dybvig, K., Hollingshead, S. K., Heath, D. G., Clewell, D. B., Sun, F., and Woodard, A. (1992). "Degenerate oligonuclotide primes for enzymatic amplification of *recA* sequences from Gram-positive bacteria and mycoplasmas." *J. Bacteriol.*, **174**: 2729-2732.

Dybvig, K., Sitaraman, R., and French, C. T. (1998). "A family of phase-variable restriction enzymes with differing specificities generated by high-frequency gene rearrangements." *Proc. Natl. Acad. Sci. USA*, **95**: 13923-13928.

Dybvig, K., and Voelker, L. L. (1996). "Molecular biology of mycoplasmas." *Annu. Rev. Microbiol.*, **50**: 25-57.

Engler-Blum, G., Meier, M., Frank, J., and Müller, G. A. (1993). "Reduction of background problems in nonradioactive northern and southern blot analyses enables higher sensitivity than ^{32}P-based hybridizations." *Anal. Biochem.*, **210**: 235-244.

Erickson, B. Z., Ross, R. F., Rose, D. L., Tully, J. G., and Bové, J. M. (1986). "*Mycoplasma hyopharyngis*, a new species from swine." *Int. J. Syst. Bacteriol.*, **36**: 55-59.

Fagan, P. K., Djordjevic, S. P., Chin, J., Eamens, G. J., and Walker, M. J. (1997). "Oral immunization of mice with attenuated *Salmonella typhimurium aroA* expressing recombinant *Mycoplasma hyopneumoniae* antigen (NrdF)." *Infect. Immun.*, **65**: 2502-2507.

Fagan, P. K., Djordjevic, S. P., Eamens, G. J., Chin, J., and Walker, M. J. (1996). "Molecular characterization of a ribonucleotide reductase (*nrdF*) gene fragment of *Mycoplasma hyopneumoniae* and assesment of the recombinant product as an experimental vaccine for enzootic pneumonia." *Inf. Imm.*, **64**: 1060-1064.

Falah, M., and Gupta, R. (1997). "Phylogenetic analysis of mycoplasmas based on Hsp70 sequences: cloning of the *dnaK* (*hsp70*) gene region of *Mycoplasma capricolum*." *Int. J. Syst. Bacteriol.*, **47**: 38-45.

Fangman, W. L. (1978). "Separation of very large DNA molecules by gel electrophoresis." *Nucleic Acids Res.*, **5**: 653-665.

Fegatella, F., Lim, J., Kjelleberg, S., and Cavicchioli, R. (1998). "Implications of rRNA operon copy number and ribosome content in the marine oligotrophic ultramicrobacterium *Sphingomonas* sp. strain RB2256." *Appl. Env. Microbiol.*, **64**: 4433-4438.

Felsenstein, J. (1993). "PHYLIP (Phylogeny Inference Package)." , Distributed by the author., University of Washington, Seattle.

Ferdows, M. S., and Barbour, A. G. (1989). "Megabase-sized linear DNA in the bacterium *Borrelia burgdorferi*, the Lyme disease agent." *Proc. Natl. Acad. Sci. USA*, **86**: 5969-5973.

Ferrell, R. V., Heidari, M. B., Wise, K. S., and McIntosh, M. A. (1989). "A *Mycoplasma* genetic element resembling prokaryotic insertion sequences." *Mol. Microbiol.*, **3**: 957-967.

Fleischmann, R. D., Adams, M. D., White, O., Clayton, R. A., Kirkness, E. F., Kerlavage, A. R., and others. (1995). "Whole genome random sequencing and assembly of *Haemophilus influenzae* Rd." *Science*, **269**: 496-512.

Fonstein, M., and Haselkorn, R. (1995). "Physical mapping of bacterial genomes." *J. Bacteriol.*, **177**: 3361-3369.

Fox, G. E., Stackebrandt, E., Hespell, R. B., Gibson, J., Maniloff, J., Dyer, T. A., and others. (1980). "The phylogeny of prokaryotes." *Science*, **209**: 457-463.

Fraser, C. M., Gocayne, J. D., White, O., Adams, M. D., Clayton, R. A., Fleischmann, R. D., and others. (1995). "The minimal gene complement of *Mycoplasma genitalium*." *Science*, **270**: 397-403.

Freeman, M. J., Armstrong, C. H., Sands-Freeman, L. L., and Lopez-Osuna, M. (1984). "Serological cross-reactivity of porcine reference antisera to *Mycoplasma hyopneumoniae*, *M. flocculare*, *M. hyorhinis* and *M. hyosynoviae* indicated by the enzyme-linked immunosorbent assay, complement fixation and indirect hemagglutination tests." *Can. J. Comp. Med.*, **48**: 202-207.

Frey, J., Haldimann, A., and Nicolet, J. (1992). "Chromosomal heterogeneity of various *Mycoplasma hyopneumoniae* field strains." *Int. J. Syst. Bacteriol.*, **42**: 275-280.

Friis, N. F. (1972). "Isolation and characterization of a new porcine mycoplasma." *Acta Vet. Scand.*, **13**: 284-286.

Friis, N. F. (1973). "The pathogenicity of *Mycoplasma flocculare*." *Acta Vet. Scand.*, **14**: 344-346.

Friis, N. F. (1975). "Some recommendations concerning primary isolation of *Mycoplasma suipneumoniae* and *Mycoplasma floccluare*." *Nord. Vet. Med.*, **27**: 337-339.

Futo, S., Seto, Y., Mitsuse, S., Mori, Y., Suzuki, T., and Kawai, K. (1995a). "Molecular cloning of a 46-kilodalton surface antigen (P46) gene from *Mycoplasma hyopneumoniae*: direct evidence of CGG codon usage for arginine." *J Bacteriol*, **177**: 1915-1917.

Futo, S., Seto, Y., Okada, M., Sato, S., Suzuki, T., Kawai, K., and others. (1995b). "Recombinant 46-kilodalton surface antigen (P46) of *Mycoplasma hyopneumoniae* expressed in *Escherichia coli* can be used for early specific diagnosis of mycoplasma pneumoniae of swine by enzyme-linke immunosorbent assay." *J. Clin. Microbiol.*, **33**: 680-683.

Gabridge, M. G., Chandler, D. K. F., and Daniels, M. J. (1985). "Pathogenicity factors in mycoplasmas and spiroplasmas." In *The Mycoplasmas IV: Mycoplasma Pathogenicity*, S. Razin and M. F. Barile, eds., Academic Press, Orlando FL, 313-351.

Gafny, R., Hyman, H. C., Razin, S., and Glaser, G. (1988). "Promoters of *Mycoplasma capricolum* ribosomal RNA operons: Identical activities but different regulation in homologous and heterologous cells." *Nucleic Acids Res.*, **16**: 61-76.

Gardiner, K., Laas, W., and Patterson, W. (1986). "Fractionation of large mammalian DNA restriction fragments using vertical pulsed field gradient gel electrophoresis." *Somatic Cell Mol. Genet.*, **12**: 185-195.

Geary, S. J., Tourtellotte, M. E., and Cameron, J. A. (1981). "Inflammatory toxin from *Mycoplasma bovis*: isolation and characterization." *Science*, **212**: 1032-1033.

Geary, S. J., and Walczak, E. M. (1985). "Isolation of a cytopathic factor from *Mycoplasma hyopneumoniae*." *Inf. Imm.*, **48**: 576-578.

Ghosh, A., Das, J., and Maniloff, J. (1977). "Lack of repair of ultraviolet light damage in *Mycoplasma gallisepticum*." *J. Mol. Biol.*, **116**: 337-344.

Glaser, G., Amikam, D., and Razin, S. (1981). "Stable RNA synthesis and its control in *Mycoplasma capricolum*." *Nucleic Acids Res.*, **9**: 3641-3646.

Göbel, U., Butler, G. H., and Stanbridge, E. J. (1984). "Comparative analysis of mycoplasmal ribosomal RNA operons." *Isr. J. Med. Sci.*, **20**: 762-764.

Goh, S. H., Potter, S., Wood, J. O., Hemmingsen, S. M., Reynolds, R. P., and Chow, A. W. (1996). "HSP60 gene sequences as universal targets for microbial species identification: studies with coagulase-negative staphylococci." *J. Clin. Microbiol.*, **34**: 818-823.

Goodwin, R. F., Pomeroy, A. P., and Whittlestone, P. (1965). "Production of enzootic pneumonia in pigs with a mycoplasma." *Vet. Rec.*, **77**: 1247-1249.

Gorton, T. S., Goh, S., and Geary, S. J. (1995). "Physical mapping of the *Mycoplasma gallisepticum* S6 genome with localization of selected genes." *J. Bacteriol.*, **177**: 259-263.

Gravius, B., Cullum, J., and Hranueli, D. (1994). "High G+C-content DNA markers for pulsed-field gel electrophoresis." *Biotechniques*, **16**: 52.

Gubler, M., Jetten, M., Lee, S. H., and Sinskey, A. (1994). "Cloning of the pyruvate kinase gene (*pyk*) of *Corynebacterium glutamicum* and site-specific inactivation of *pyk* in a lysine producing *Corynebacterium lactofermentum* strain." *App. Env. Microbiol.*, **60**: 2494-2500.

Gundersen, D. E., Lee, I.-M., Schaff, D. A., Harrison, N. A., Chang, C. J., Davis, R. E., and Kingsbury, D. T. (1996). "Genomic diversity and differentiation among phytoplasma strains in 16S rRNA groups I (aster yellows and related phytoplasmas) and III (X-disease and related phytoplasmas)." *Int. J. Syst. Bacteriol.*, **46**: 64-75.

Gupta, R. (1998). "Protein phylogenies and signature sequences: a reappraisal of evolutionary relationships among archaebacteria, eubacteria, and eukaryotes." *Microbiol. Mol. Biol. Rev.*, **62**: 1435-1491.

Gurrieri, S., Rizzarelli, E., Beach, D., and Bustamante, C. (1990). "Imaging of kinked configurations of DNA molecules undergoing orthogonal field alternating gel electrophoresis by fluorescence microscopy." *Biochemistry*, **29**: 3396-3401.

Hagerman, P. J. (1986). "Sequence directed curvature of DNA." *Nature*, **321**: 449-450.

Hagerman, P. J. (1988). "Flexibility of DNA." *Ann. Rev. Biophys. Biophys. Chem.*, **17**: 265-286.

Haldimann, A., Nicolet, J., and Frey, J. (1993). "DNA sequence determination and biochemical analysis of immunogenic protein P36, the lactate dehydrogenase (LDH) of *Mycoplasma hyopneumoniae*." *J. Gen. Microbiol.*, **139**: 317-323.

Hanish, J., and McClelland, M. (1990). "Methylase-limited partial *Not*I cleavage for physical mapping of genome DNA." *Nucleic Acids Res.*, **18**: 3287-3291.

Hannan, P. C. T., Windsor, G. D., DeJong, A., Schmeer, N., and Stegemann, M. (1997). "Comparative susceptibilities of various animal-pathogenic mycoplasmas to fluoroquinolones." *Antimicrob. Agents Chemother.*, **41**: 2037-2040.

Harasawa, R., Asada, K., and Kato, I. (1995). "A novel repetitive sequence from *Mycoplasma hyopneumoniae*." *J. Vet. Med. Sci.*, **57**: 557-558.

Heller, C., and Pohl, F. M. (1989). "A systematic study of field inversion gel electrophoresis." *Nucleic Acids Res.*, **17**: 5989-6003.

Heller, C., and Pohl, F. M. (1990). "Field inversion gel electrophoresis with different pulse time ramps." *Nucleic Acids Res.*, **18**: 6299-6304.

Henrich, B., Feldman, R.-C., and Hadding, U. (1993). "Cytadhesins of *Mycoplasma hominis*." *Infect. Immun.*, **61**: 2945-2951.

Henrich, B., Kitzerow, A., Feldman, R.-C., Schaal, H., and Hadding, U. (1996). "Repetitive elements of *Mycoplasma hominis* adhesin P50 can be differentiated by monoclonal antibodies." *Infect. Immun.*, **64**: 4027-4034.

Herrmann, R. (1992). "Genome structure and organization." In *Mycoplasmas: Molecular Biology and Pathogenesis*, J. Maniloff, R. N. McElhaney, L. R. Finch, and J. B. Baseman, eds., ASM Press, Washington D.C., 157-168.

Hilbert, H., Himmelreich, R., Plagens, H., and Herrmann, R. (1996). "Sequence analysis of 56 kb from the genome of the bacterium *Mycoplasma pneumoniae* comprising the dnaA region, the atp operon and a cluster of ribosomal protein genes." *Nucleic Acids Res.*, **24**: 628-639.

Himmelreich, R., Hilbert, H., Plagens, H., Pirkl, E., Li, B.-C., and Herrmann, R. (1996). "Complete sequence analysis of the genome of the bacterium *Mycoplasma pneumoniae*." *Nucleic Acids Res.*, **24**: 4420-4449.

Himmelreich, R., Plagens, H., Hilbert, H., Reiner, B., and Herrmann, R. (1997). "Comparative analysis of the genomes of the bacteria *Mycoplasma pneumoniae* and *Mycoplasma genitalium*." *Nucleic Acids Res.*, **25**: 701-712.

Howard, C. J., and Taylor, G. (1985). "Humoral and cell-mediated immunity." In *The Mycoplasmas IV: Mycoplasma Pathogenicity*, S. Razin and M. F. Barile, eds., Academic Press, Orlando FL, 259-292.

Hsu, T., Artiushin, S., and Minion, F. C. (1997). "Cloning and functional analysis of the P97 swine cilium adhesin gene of *Mycoplasma hyopneumoniae*." *J. Bacteriol.*, **179**: 1317-1323.

Hsu, T., and Minion, F. C. (1998). "Molecular analysis of the P97 cilium adhesin operon of *Mycoplasma hyopneumoniae*." *Gene*, **214**: 13-23.

Huang, Y., Robertson, J. A., and Stemke, G. W. (1995). "An unusual rRNA gene organization in *Mycoplasm fermentas* (incognitus strain)." *Can. J. Microbiol.*, **41**: 424-427.

Huang, Y., and Stemke, G. W. (1992). "Construction of the physical maps of *Mycoplasma hyopneumoniae* and *Mycoplasma flocculare* and the location of rRNA genes on these maps." *Can. J. Microbiol.*, **38**: 659-663.

International Committee on Systematic Bacteriology Subcommittee on the Taxonomy of Mollicutes. (1990). "Minutes of the interim meeting, 7 and 8 July 1990, Istanbul, Turkey." *Int. J. Syst. Bacteriol*, **41**: 333-336.

International Committee on Systematic Bacteriology Subcommittee on the Taxonomy of Mollicutes. (1992). "Minutes of the interim meeting, 1 and 1 August 1992, Ames, Iowa." *Int. J. Syst. Bacteriol*, **43**: 394-397.

Jensen, P. T., Wolstrup, C., and Friis, N. F. (1978). "Utilisation of glucose by *Mycoplasma suipneumoniae* and *Mycoplasma flocculare*." *Acta. Vet. Scand.*, **19**: 179-183.

Johansson, K.-E., Mattsson, J. G., Jacobsson, K., Fernandez, C., Bergström, K., Bölske, G., Wallgren, P., and Göbel, U. B. (1992). "Specificity of oligonucleotide probes complementary to evolutionarily variable regions of 16S rRNA from *Mycoplasma hyopneumoniae* and *Mycoplasma hyorhinis*." *Res. Vet. Sci.*, **52**: 195-204.

Jumas-Bilak, E., Michaux-Charachon, S., Bourg, G., Ramuz, M., and Allardet-Servent, A. (1998). "Unconventional genomic organization in the alpha subgroup of the *Proteobacteria*." *J. Bacteriol.*, **180**: 2749-2755.

Kamla, V., Henrich, B., and Hadding, U. (1996). "Phylogeny based on elongation factor Tu reflects the phenotypic features of mycoplasmas better than that based on 16S rRNA." *Gene*, **171**: 83-87.

Keeler, C. L., Hnatow, L. L., Whetzel, P., and Dohms, J. E. (1996). "Cloning and characterization of a putative cytadhesisn gene (*mgc1*) from *Mycoplasma gallisepticum*." *Infect. Immun.*, **64**: 1541-1547.

Kerr, A. R. W., Pedan, J. F., and Sharp, P. M. (1997). "Systematic base composition variation around the genome of *Mycoplasma genitalium*, but not *Mycoplasma pneumoniae*." *Mol. Microbiol.*, **25**: 1177-1179.

Kieser, H., Kieser, T., and Hopwood, D. (1992). "A combined genetic and physical map of the *Streptomyces coelicolor* A3(2) chromosome." *J. Bacteriol.*, **174**: 5496-5507.

Kim, U.-J., Shizuya, H., Jong, P. J. d., Birren, B., and Simon, M. I. (1992). "Stable propagation of cosmid sized human DNA inserts in an F factor based vector." *Nucleic Acids Res.*, **20**: 1083-1085.

King, K. W., and Dybvig, K. (1993). "Mycoplasmal cloning vectors derived from plasmid pKMK1." *Plasmid*, **31**(49-59).

King, K. W., Faulds, D. H., Rosey, E. L., and Yancey Jr., R. J. (1997). "Characterization of the gene encoding Mhp1 from *Mycoplasma hyopneumoniae* and examination of Mhp1's vaccine potential." *Vaccine*, **15**: 25-35.

Kirchhoff, H., and Flossdorf, J. (1987). "Determination of the guanosine-plus-cytosine content of *Mollicutes* by isopycnic gradient centrifugation." *Int. J. Syst. Bacteriol.*, **37**: 454-455.

Kobisch, M., and Friis, N. F. (1996). "Swine mycoplasmoses." *Rev. Sci. Tech. Off. Int. Epiz.*, **15**: 1569-1605.

Kobisch, M., Quillien, L., Tillon, J. P., and Wróblewski, H. (1987). "The *Mycoplasma hyopneumoniae* plasma membrane as a vaccine against porcine enzootic pneumonia." *Ann. Inst. Pasteur/Immunol.*, **138**: 693-705.

Kohara, Y., Akiyama, K., and Isono, K. (1987). "The physical map of the whole *E. coli* chromosome: Application of a new strategy for rapid analysis and sorting of a large genomic library." *Cell*, **50**: 495-508.

Krause, D. C. (1996). "*Mycoplasma pneumoniae* cytadherence: unravelling the tie that binds." *Mol. Microbiol.*, **20**: 247-253.

Krause, D. C., and Taylor-Robinson, D. (1992). "Mycoplasmas which infect humans." In *Mycoplasmas: Molecular Biology and Pathogenesis*, J. Maniloff, R. N. McElhaney, L. R. Finch, and J. B. Baseman, eds., ASM Press, Washington D.C., 417-444.

Kristensen, B., Paroz, P., Nicolet, J., Wanner, M., and Weck, A. L. d. (1981). "Cell mediated and humoral immune response in swine after vaccination and natural infection with *Mycoplasma hyopneumoniae*." *Am. J. Vet. Res.*, **42**: 784-788.

Kuempel, P. L., Pelletier, A. J., and Hill, T. M. (1990). "*tus* and the terminators: inhibition of replication in *Escherichia coli*." In *The Bacterial Chromosome*, K. Drlica and M. Riley, eds., American Society for Microbiology, Washington DC, 297-306.

Kuspa, A., Vollrath, D., Cheng, Y., and Kaiser, D. (1989). "Physical mapping of the *Myxococcus xanthus* genome by random cloning in yeast artificial chromosomes." *Proc. Natl. Acad. Sci. USA*, **86**: 8917-8921.

Ladefoged, S. A., and Christiansen, G. (1992). "Physical and genetic mapping of the genomes of five *Mycoplasma hominis* strains by pulsed-field gel electrophoresis." *J. Bacteriol.*, **174**: 2199-2207.

Ladefoged, S. A., and Christiansen, G. (1994). "Sequencing analysis reveals a unique gene organization in the *gyrB* region of *Mycoplasma hominis*." *J. Bacteriol.*, **176**: 5835-5842.

Lalande, M., Noolandi, J., Turmel, C., Brousseau, R., Rousseau, J., and Slater, G. W. (1988). "Scrambling of bands in gel electrophoresis of DNA." *Nucleic Acids Res.*, **16**: 5427-5437.

Lane, D. J., Pace, B., Olsen, G. J., Stahl, D. A., Sogin, M. L., and Pace, N. R. (1985). "Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses." *Proc. Natl. Acad. Sci. USA*, **82**: 6955-6959.

Leblond, P., Fischer, G., Francou, F.-X., Berger, F., Guérineau, M., and Decaris, B. (1996). "The unstable region of *Streptomyces ambofaciens* includes 210kb terminal inverted repeats flanking the extremities of the linear chromosomal DNA." *Mol. Microbiol.*, **19**: 261-271.

Lederberg, J., and Clair, J. S. (1958). "Protoplasts and L-type growth of bacteria." *J. Bacteriol.*, **75**: 143-160.

Lee, J. J., and Smith, H. O. (1988). "Sizing of the *Haemophilus influenzae* Rd genome by pulsed-field agarose gel electrophoresis." *J. Bacteriol.*, **170**: 4402-4405.

Lee, J. J., Smith, H. O., and Redfield, R. J. (1989). "Organization of the *Haemophilus influenzae* Rd genome." *J. Bacteriol.*, **171**: 3016-3024.

Ligon, J. V., and Kenny, G. E. (1991). "Virulence of ureaplasmal urease for mice." *Infect. Immun.*, **59**: 1170-1171.

Lin, Y.-S., Kieser, H. M., Hopwood, D. A., and Chen, C. W. (1993). "The chromosomal DNA of *Streptomyces lividans* 66 is linear." *Mol. Microbiol.*, **10**: 923-933.

Lo, S.-C. (1992). "Mycoplasmas and AIDS." In *Mycoplasmas: Molecular Biology and Pathogenesis*, J. Maniloff, R. N. McElhaney, L. R. Finch, and J. B. Baseman, eds., ASM Press, Washington D.C., 525-545.

Locke, J., Rairdan, G., McDermid, H., Nash, D., Pilgrim, D., Bell, J., and others. (1996). "Cross-screening: a new method to assemble clones rapidly and unambiguously into contigs." *Genome Res.*, **6**(155-165).

Lucier, T. S., Hu, P.-Q., Peterson, S. N., Song, X.-Y., Miller, L., Heitzman, K., and others. (1994). "Construction of an ordered genomic library of *Mycoplasma genitalium*." *Gene*, **150**: 27-34.

Mahan, M. J., Segall, A. M., and Roth, J. R. (1990). "Recombination events that rearrange the chromosome: barriers to inversion." In *The Bacterial Chromosome*, K. Drlica and M. Riley, eds., American Society for Microbiology, Washington DC, 341-349.

Maidak, B. L., Larsen, N., McCaughy, M. J., Overbeek, R., Olsen, G. J., Fogel, K., Blandy, J., and Woese, C. R. (1994). "The Ribosomal Database Project." *Nucleic Acids Res.*, **22**: 3485-3487.

Maniloff, J. (1989). "Anomalous values of *Mycoplasma* genomes sizes determined by pulse-field gel electrophoresis." *Nucleic Acids Res.*, **17**: 1268.

Maniloff, J. (1992a). "Mycoplasma viruses." In *Mycoplasmas: Molecular Biology and Pathogenesis*, J. Maniloff, R. N. McElhaney, L. R. Finch, and J. B. Baseman, eds., ASM Press, Washington D.C., 41-59.

Maniloff, J. (1992b). "Phylogeny of Mycoplasmas." In *Mycoplasmas: Molecular Biology and Pathogenesis*, J. Maniloff, R. N. McElhaney, L. R. Finch, and J. B. Baseman, eds., ASM Press, Washington D.C., 549-559.

Marais, A., Bové, J. M., and Renoudin, J. (1996). "*Spiroplasma citri* virus SpV1-derived cloning vector: deletion formation by illegitimate and homologous recombination in a spiroplasmal host which probably lacks a functional *recA* gene." *J. Bacteriol.*, **178**: 862-870.

Mare, C. J., and Switzer, W. P. (1965). "New species: *Mycoplasma hyopneumoniae*, a causative agent of virus pig pneumonia." *Vet. Med.*, **60**: 841-846.

Margerrison, E. E., Hopewell, R., and Fisher, L. M. (1992). "Nucleotide sequence of the *Staphylococcus aureus gyrB-gyrA* locus encoding the DNA gyrase A and B proteins." *J. Bacteriol.*, **174**: 1596-1603.

Mayr, E. (1998). "Two empires or three?" *Proc. Natl. Acad. Sci.*, **95**: 9720-9723.

McGarrity, G. J., and Kotani, H. (1985). "Cell culture mycoplasmas." In *The Mycoplasmas IV: Mycoplasma Pathogenicity*, S. Razin and M. F. Barile, eds., Academic Press, Orlando FL, 353-390.

McGee, Z. A., Rogul, M., and Wittler, R. G. (1967). "Molecular genetic studies of relationships among mycoplasma, L-forms, and bacteria." *Ann. N.Y. Acad. Sci.*, **143**: 21-30.

Mebus, C. A., and Underdahl, N. R. (1977). "Scanning electron microscopy of trachea and bronchi from gnotobiotic pigs inoculated with *Mycoplasma hyopneumoniae*." *Am. J. Vet. Res.*, **38**: 1249-1254.

Meyling, A., and Friis, N. F. (1972). "Serological identification of a new porcine mycoplasma species, *M. flocclare*." *Acta Vet. Scand.*, **13**: 287-289.

Miyata, M., Sano, K.-I., Okada, R., and Fukumura, T. (1993). "Mapping of replication initiaion site in *Mycoplasma capricolum* genome by two-dimensional gel-electrophoretic analysis." *Nucleic Acids Res.*, **21**: 4816-4823.

Miyata, M., Wang, L., and Fukumura, T. (1991). "Physical mapping of the *Mycoplasma capricolum* genome." *FEMS Microbiol. Lett.*, **79**: 329-334.

Mizrahi, V., Huberts, P., Dawes, S. S., and Dudding, L. R. (1993). "A PCR method for the sequence analysis of the *gyrA*, *polA*, and *rnhA* gene segments from mycobacteria." *Gene*, **136**: 287-290.

Morowitz, H. J. (1984). "The completeness of molecular biology." *Isr. J. Med. Sci.*, **20**: 750-753.

Morowitz, H. J., and Wallace, D. C. (1973). "Genome size and life cycle of the mycoplasmas." *Ann. N.Y. Acad. Sci.*, **225**: 63-73.

Mushegian, A. R., and Koonin, E. V. (1996). "A minimal gene set for cellular life derived by comparison of complete bacterial genomes." *Proc. Natl. Acad. Sci. USA*, **93**: 10268-10273.

Muto, A. (1987). "The genome structure of *Mycoplasma capricolum*." *Isr. J. Med. Sci.*, **23**: 334-341.

Muto, A., Andachi, Y., Yamao, F., Tanaka, R., and Osawa, S. (1992). "Transcription and translation." In *Mycoplasmas: Molecular Biology and Pathogenesis*, J. Maniloff, R. N. McElhaney, L. R. Finch, and J. B. Baseman, eds., ASM Press, Washington D.C., 331-347.

Muto, A., Andachi, Y., Yuzawa, H., Yamao, F., and Osawa, S. (1990). "The organization and evolution of transfer RNA genes of *Mycoplasma capricolum*." *Nucleic Acids Res.*, **18**: 5037-5043.

Muto, A., Kawauchi, Y., Yamao, F., and Osawa, S. (1984). "Preferential use of A- and U-rich codons for *Mycoplasma capricolum* ribosomal proteins S8 and L6." *Nucleic Acids Res.*, **12**: 8209-8217.

Neimark, H. C., and Lange, C. S. (1990). "Pulse-field electrophoresis indicates full-length mycoplasma chromosomes range widely in size." *Nucleic Acids Res.*, **18**: 5443-5448.

Neimark, H. C., and London, J. (1982). "Origins of mycoplasmas: sterol-nonrequiring mycoplasmas evolved from streptococci." *J. Bacteriol.*, **150**: 1259-1265.

Oba, T., Andachi, Y., Muto, A., and Osawa, S. (1991). "CGG: an unassigned or nonsense codon in *Mycoplasma capricolum*." *Proc. Natl. Acad. Sci.*, **88**: 921-925.

Ogasawara, N., Fujita, M. Q., Moriya, S., Fukuoka, T., Hirano, M., and Yoskikawa, H. (1990). "Comparative anatomy of *oriC* of eubacteria." In *The Bacterial Chromosome*, K. Drlica and M. Riley, eds., American Society for Microbiology, Washington DC, 287-295.

Page, R. D. M. (1996). "TREEVIEW: an application to view phylogenetic trees on personal computers." *Comp. App. Biosci.*, **12**: 357-358.

Peterson, S. N., Hu, P.-C., Bott, K. F., and Hutchison III, C. A. (1993). "A survey of the *Mycoplasma genitalium* genome by using random sequencing." *J. Bacteriol.*, **175**: 7918-7930.

Poddar, S. K., and Maniloff, J. (1989). "Determination of microbial genome sizes by two-dimensional denaturing gradient gel electrophoresis." *Nucleic Acids Res.*, **17**: 2889-2895.

Pointon, A. M., Byrt, D., and Heap, P. (1985). "Effect of enzootic pneumonia of pigs on growth performance." *Aus. Vet. J.*, **62**: 13-18.

Pollack, J. D. (1992). "Carbohydrate metabolism and energy conservation." In *Mycoplasmas: Molecular Biology and Pathogenesis*, J. Maniloff, R. N. McElhaney, L. R. Finch, and J. B. Baseman, eds., ASM Press, Washington D.C., 181-200.

Pollack, J. D. (1997). "Mycoplasma genes: a case for reflective annotation." *Trends Microbiol.*, **5**: 413-419.

Pyle, L. E., Corcoran, L. N., Cocks, B. G., Bergemann, A. D., Whitley, J. C., and Finch, L. R. (1988). "Pulsed-field electrophoresis indicates larger-than-expected sizes for mycoplasma genomes." *Nucleic Acids Res.*, **16**: 6015-6025.

Pyle, L. E., and Finch, L. R. (1988). "A physical map of *Mycoplasma mycoides* subspecies *mycoides* Y with some functional loci." *Nucleic Acids Res.*, **16**: 6027-6039.

Pyle, L. E., Taylor, T., and Finch, L. R. (1990). "Genomic maps of some strains within the *Mycoplasma mycoides* cluster." *J. Bacteriol.*, **172**: 7265-7268.

Razin, S. (1992). "Mycoplasma Taxonomy and Ecology." In *Mycoplasmas: Molecular Biology and Pathogenesis*, J. Maniloff, R. N. McElhaney, L. R. Finch, and J. B. Baseman, eds., ASM Press, Washington D.C., 3-22.

Razin, S., and Freundt, E. A. (1984). "The mycoplasmas." In *Bergey's manual of systematic bacteriology*, N. R. Kreig and J. G. Holt, eds., The Williams & Wilkins, Co., Baltimore, 740-793.

Razin, S., Yogev, D., and Naot, Y. (1998). "Molecular biology and pathogenicity of mycoplasmas." *Microbiol. Mol. Biol. Rev.*, **62**: 1094-1156.

Renbaum, P., Abrahamove, D., Fainsod, A., Wilson, G. G., Rottem, S., and Razin, A. (1990). "Cloning, characterization, and expression in *Escherichia coli* of the gene coding for CpG DNA methylase from *Spiroplasma sp.* strain MQ1(M-SssI)." *Nucleic Acids Res.*, **18**: 1145-1151.

Ro, L. H., and Ross, R. F. (1983). "Comparison of *Mycoplasma hyopneumoniae* strains by serologic methods." *Am. J. Vet. Res.*, **44**: 2087-2094.

Roberts, D. D., Olson, L. D., Barile, M. F., Ginsburg, V., and Krivans, H. C. (1989). "Silaic acid-dependant adhesion of *Mycoplasma pneumoniae* to purified glycoproteins." *J. Biol. Chem.*, **264**: 9289-9293.

Robertson, J. A., Pyle, L. E., Stemke, G. W., and Finch, L. R. (1990). "Human ureaplasmas show diverse genome sizes by pulsed-field electrophoresis." *Nucleic Acids Res.*, **18**: 1451-1455.

Robertson, J. A., Vekris, A., Bebear, C., and Stemke, G. W. (1993). "Polymerase chain reaction using 16S rRNA gene sequences distinguises the two biovars of *Ureaplasma urealyticum*." *J. Clin. Micrb.*, **31**: 824-830.

Rodwell, A. W. (1983). "Defined and partly defined media." In *Methods in Mycoplasmology*, S. Razin and J. G. Tully, eds., Academic Press, New York, 163-172.

Rodwell, A. W., and Mitchell, A. (1979). "Nutrition, growth, and reproduction." In *The Mycoplasmas I: Cell Biology*, M. F. Barile and S. Razin, eds., Academic Press, New York, 103-139.

Romano, N., Tolone, G., Ajello, F., and LaLicata, R. (1980). "Adenosine 5'-triphosphate synthesis induced by urea hydrolysis in *Ureaplasma urealyticum*." *J. Bacteriol.*, **144**: 830-832.

Rose, D. L., Tully, J. G., and Wittler, R. G. (1979). "Taxonomy of some swine mycoplasmas: *Mycoplasma suipneumoniae* Goodwin et al. 1965, a later, objective synonym of *Mycoplasma hyopneumoniae* Mare and Switzer 1965, and the status of *Mycoplasma flocculare* Meyling and Friis 1972." *Int. J. Syst. Bacteriol.*, **29**: 83-91.

Rosengarten, R., Theiss, P. M., Yogev, D., and Wise, K. S. (1993). "Antigenic variation in *Mycoplasma hyorhinis*: increased repertoire of variable lipoproteins expanding surface diversity and structural complexity." *Infect. Immun.*, **61**: 2224-2228.

Rosengarten, R., and Wise, K. S. (1991). "The Vlp system of *Mycoplasma hyorhinis*: combinatorial expression of distinct size variant lipoproteins generating high-frequency surface antigenic variation." *J. Bacteriol.*, **173**: 4782-4793.

Ross, R. F. (1992). "Mycoplasmal diseases." In *Diseases of Swine*, A. D. Leman, B. E. Straw, W. L. Mengeling, S. D'Allaire, and D. J. Taylor, eds., Iowa State University Press, Ames, IA, 537-551.

Ross, R. F., and Cox, D. F. (1988). "Evaluation of tiamulin for the treatment of mycoplasmal pneumonia in swine." *J. Am. Vet. Med. Assoc.*, **193**: 441-446.

Ross, R. F., and Duncan, J. R. (1970). "*Mycoplasma hyosynoviae* arthritis of swine." *J. Am. Vet. Med. Assoc.*, **157**: 1515-1518.

Ross, R. F., and Karmon, J. A. (1970). "Heterogeneity among strains of *Mycoplasma granularum* and identification of *Mycoplasma hyosynoviae*, sp. n." *J. Bacteriol.*, **103**: 707-713.

Ross, R. F., Zimmermann-Erickson, B. J., and Young, T. F. (1984). "Characteristics of protective activity of *Mycoplasma hyopneumoniae* vaccine." *Am. J. Vet. Res.*, **45**: 1899-1905.

Roux, K. H. (1994). "Using mismatched primer-template pairs in touchdown PCR." *Biotechniques*, **16**: 812-814.

Ruland, K., Himmelreich, R., and Herrmann, R. (1994). "Sequence divergence in the ORF6 gene of *Mycoplasma pneumoniae*." *J. Bacteriol.*, **176**: 5202-5209.

Ruuth, E., and Praz, F. (1989). "Interactions between mycoplasmas and the immune system." *Immunol. Rev.*, **112**: 134-160.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbour Laboratory Press, Cold Spring Harbour, NY.

Sano, K.-I., and Miyata, M. (1994). "The *gyrB* gene lies opposite from the replication origin ont he circular chromosome of *Mycoplasma capricolum*." *Gene*, **151**: 181-183.

Schneider, B., Gibb, K. S., and Seemuller, E. (1997). "Sequence and RFLP analysis of the elongation factor TU used in differentiation and classification of phytoplasmas." *Microbiolgoy*, **143**: 3381-3389.

Schwartz, D. C., and Cantor, C. R. (1984). "Separation of yeast chromosome-sized DNAs by pulsed-field gradient gel electrophoresis." *Cell*, **37**: 67-75.

Simecka, J. W., Davis, J. K., Davidson, M. K., Ross, S. E., Städtlander, C. T. K.-H., and Cassel, G. H. (1992). "Mycoplasma diseases in animals." In *Mycoplasmas: Molecular Biology and Pathogenesis*, J. Maniloff, R. N. McElhaney, L. R. Finch, and J. B. Baseman, eds., ASM Press, Washington D.C., 391-416.

Skamrov, A., Goldman, M., Klasova, J., and Beabealasvilli, R. (1995). "*Mycoplasma gellisepticum* 16S rRNA genes." *FEMS Microbiol. Lett.*, **128**: 321-325.

Smart, C. D., Schneider, B., Blomquist, C. L., Guerra, L. J., Harrison, N. A., Ahrens, U., and others. (1996). "Phytoplasma-specific PCR primers based on sequences of the 16S-23S rRNA spacer region." *Appl. Environ. Microbiol.*, **62**: 2988-2993.

Smiley, B. K., and Minion, F. C. (1993). "Enhanced readthrough of opal (UGA) codons and production of *Mycoplasma pneumoniae* P1 epitopes in *Escherichia coli*." *Gene*, **134**: 33-40.

Smith, C. L., Econome, J. G., Schutt, A., Klco, S., and Cantor, C. R. (1987). "A physical map of the *Escherichia coli* K12 genome." *Science*, **236**: 1448-1453.

Somerson, N. L., and Cole, B. C. (1979). "The mycoplasmas flora of human and nonhuman primates." In *The Mycoplasmas Vol. 2: Human and Animal Mycoplasmas*, J. G. Tully and R. F. Whitcomb, eds., Academic Press, New York, 191-216.

Somerson, N. L., Walls, B. E., and Chanock, R. M. (1965). "Hemolysin of *Mycoplasma pneumoniae*: tentative identification as a peroxide." *Science*, **150**: 226-228.

Southern, E. M., Anand, R., Brown, W. R. A., and Fletcher, D. S. (1987). "A model for the separation of large DNA molecules by crossed field gel electrophoresis." *Nucleic Acids Res.*, **15**: 5925-5943.

Stemke, G. W., Huang, Y., Laigret, F., and Bové, J. M. (1994a). "Cloning the ribosomal RNA operons of *Mycoplasma flocculare* and comparison with those of *Mycoplasma hyopneumoniae*." *Microbiology*, **140**: 857-860.

Stemke, G. W., Laigret, F., Grau, O., and Bové, J. M. (1992). "Phylogenetic relationships of three porcine mycoplasmas, *Mycoplasma hyopneumoniae*, *Mycoplasma flocculare*, and *Mycoplasma hyorhinis*, and complete 16S rRNA sequence of *M. flocculare*." *Int. J. Syst. Bacteriol.*, **42**: 220-225.

Stemke, G. W., McIntyre, D. J., Roy, K. L., Stemler, M. E., and Robertson, J. A. (1985). "Guanine-plus-cytosine composition of and deoxyribonucleic acid hybridization comparisons between *Mycoplasma hyopneumoniae* and *Mycoplasma flocculare*." *Int. J. Syst. Bacteriol.*, **35**: 527-529.

Stemke, G. W., Phan, R., Young, T. F., and Ross, R. F. (1994b). "Differentiation of *Mycoplasma hyopneumoniae*, *M. flocculare*, and *M. hyorhinis* on the basis of amplification of a 16S rRNA gene sequence." *Am. J. Vet. Res.*, **55**: 81-84.

Stemke, G. W., and Robertson, J. A. (1990). "The growth response of *Mycoplasma hyopneumoniae* and *Mycoplasma flocculare* based upon ATP-dependent luminometry." *Vet. Microbiol.*, **24**: 135-142.

Strasser, M., Abiven, P., Kobisch, M., and Nicolet, J. (1992). "Immunological and pathological reactions in piglets experimentally infected with *Mycoplasma hyopneumoniae* and/or *Mycoplasma flocculare*." *Vet. Immun. Immunopath.*, **31**: 141-153.

Strasser, M., Frey, J., Bestetti, G., Kobisch, M., and Nicolet, J. (1991). "Cloning and expression of a species-specific early immunogenic 36-kilodalton protein of *Mycoplasma hyopneumoniae* in *Escherichia coli*." *Infect. Immun.*, **59**: 1217-1222.

Su, C. J., and Baseman, J. B. (1990). "Genome size of *Mycoplasma genitalium*." *J. Bacteriol*, **172**: 4705-4707.

Suwanto, A., and Kaplan, S. (1989). "Physical and genetic mapping of teh *Rhodobacter sphaeroides* 2.4.1. genome: presence of two unique circular chromosomes." *J. Bacteriol.*, **171**: 5850-5859.

Suzuki, Y., Mori, T., Miyata, Y., and Yamada, T. (1987). "The number of ribosomal RNA genes in *Mycobacterium lepraemurium*." *FEMS Microbiol. Lett.*, **44**: 73-76.

Taschke, C., and Herrmann, R. (1986). "Analysis of transcription and processing signal of the 16S-23S rRNA operon of *Mycoplasma hyopneumoniae*." *Mol. Gen. Genet.*, **205**: 434-441.

Taschke, C., Klinkert, M. Q., Wolter, J., and Herrmann, R. (1986). "Organization of the ribosomal RNA genes in *M. hyopneumoniae*: the 5S rRNA gene is separated from the 16S and 23S rRNA genes." *Mol. Gen. Genet.*, **205**: 428-433.

Taylor, M. A., Ferrell, R. V., Wise, K. S., and McIntosh, M. A. (1988). "Reiterated DNA sequences defining genomic diversity within the species *Mycoplasma hyorhinis*." *Mol. Microbiol.*, **2**: 665-672.

ter Laak, E. A., Pijpers, A., Noordergraaf, J. H., Schoevers, E. C., and Verheijden, J. H. M. (1991). "Comparison of methods for in vitro testing of susceptibility of porcine *Mycoplasma* species to antimicrobial agents." *Antimicrob. Agents Chemother.*, **35**: 228-233.

Tigges, E., and Minion, F. C. (1994). "Physical map of *Mycoplasma gallisepticum*." *J. Bacteriol.*, **176**: 4157-4159.

Tindall, K. R., and Kunkel, T. A. (1988). "Fidelity of DNA synthesis by the *Thermus aquaticus* DNA polymerase." *Biochemistry*, **27**: 6008-6013.

Tryon, V. V., and Baseman, J. B. (1992). "Pathogenic determinants and mechanisms." In *Mycoplasmas: Molecular Biology and Pathogenesis*, J. Maniloff, R. N. McElhaney, L. R. Finch, and J. B. Baseman, eds., ASM Press, Washington D.C., 457-471.

Tully, J. G., Bové, J. M., Laigret, F., and Whitcomb, R. F. (1993). "Revised taxonomy of the class *Mollicutes*: proposed elevation of a monophyletic cluster of arthropod-associated mollicutes to ordinal rank (*Enteroplasmatales* ord. nov.), with provision for Familial rank to separate species with nonhelical morphology (*Enteroplasmataceae* fam. nov.) from helical species (*Spiroplasmataceae*), and emended descriptions of the order *Mycoplasmatales*, family *Mycoplasmataceae*." *Int. J. Syst. Bacteriol.*, **43**: 378-385.

Turmel, C., Brassard, E., Forsyth, R., Hood, K., Slater, G. W., and Noolandi, J. (1990). "High-resolution zero integrated field electrophoresis of DNA." In *Electrophoresis of Large DNA Molecules: Theory and Applications*, E. Lai and B. Birren, eds., Cold Spring Harbor Laboratory Press, Cold Spring Harbor NY, 101-132.

Wake, R. G. (1990). "Termination of chromosome replication in *Bacillus subtilis*." In *The Bacterial Chromosome*, K. Drlica and M. Riley, eds., American Society for Microbiology, Washington DC, 307-312.

Weisburg, W. G., Tully, J. G., Rose, D. L., Petzel, J. P., Oyaizu, H., Yang, D., and others. (1989). "A phylogenetic analysis of the mycoplasmas: basis for their classification." *J. Bacteriol.*, **171**: 6455-6467.

Wenzel, R., and Herrmann, R. (1988). "Physical mapping of the *Mycoplasma pneumoniae* genome." *Nucleic Acids Res.*, **16**: 8323-8336.

Wenzel, R., and Herrmann, R. (1989). "Cloning of the complete *Mycoplasma pneumoniae* genome." *Nucleic Acids Res.*, **17**: 7029-7043.

Wenzel, R., Pirkl, E., and Herrmann, R. (1992). "Construction of an *Eco*RI restriction map of *Mycoplasma pneumoniae* and localization of selected genes." *J. Bacteriol.*, **174**: 7289-7296.

White, T. J., Bruns, T., Lee, S., and Taylor, J. (1990). "Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenies." In *PCR Protocols: A Guide to Methods and Applications*, M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White, eds., Academic Press Inc., San Diego, 315-322.

Whitley, J. C., Muto, A., and Finch, L. R. (1991). "A physical map for *Mycoplasma capricolum* Cal. kid with loci for all known tRNA species." *Nucleic Acids Res.*, **19**: 399-400.

Williams, M. V., and Pollack, J. D. (1990). "A mollicute (mycoplasma) DNA repair enzyme: purification and characterization of uracil-DNA glycosylase." *J. Bacteriol.*, **172**: 2979-2985.

Wilton, J. L., Scarman, A. L., Walker, M. J., and Djordjevic, S. P. (1998). "Reiterated repeat region variability in the ciliary adhesin gene of *Mycoplasma hyopneumoniae*." *Microbiology*, **144**: 1931-1943.

Wise, K. S., and Kim, M. F. (1987). "Major membrane surface proteins of *Mycoplasma hyopneumoniae* selectively modified by covalently bound lipid." *J. Bacteriol.*, **169**: 5546-5555.

Woese, C. R. (1987). "Bacterial Evolution." *Microbiol. Rev.*, **51**: 221-271.

Woese, C. R., and Fox, G. E. (1977). "Phylogenetic structure of the prokaryotic domain: the primary kingdoms." *Proc. Natl. Acad. Sci. USA*, **74**: 5088-5090.

Woese, C. R., Kandler, O., and Wheelis, M. L. (1990). "Towards a natural system of organisms: proposal for the domains Archaea, Bacteria, and Eucarya." *Proc. Natl. Acad. Sci. USA*, **87**: 4576-4579.

Woese, C. R., Maniloff, J., and Zablen, L. B. (1980). "Phylogenetic analysis of the mycoplasmas." *Proc. Natl. Acad. Sci. USA*, **77**: 494-498.

Woese, C. R., Stackebrandt, E., and Ludwig, W. (1985). "What are mycoplasmas: the relationship of tempo and mode in bacterial evolution." *J. Mol. Evol.*, **21**: 305-316.

Yamao, F., Muto, A., Kawauchi, Y., Iwami, M., Iwagami, S., Azumi, Y., and Osawa, S. (1985). "UGA is read as tryptophan in *Mycoplasma capricolum*." *Proc. Natl. Acad. Sci. USA*, **82**: 2306-2309.

Ye, F., Laigret, F., and Bové, J.-M. (1994a). "A physical and genomic map of the prokaryote *Spiroplasma melliferum* and its comparison with the *Spiroplasma citri* map." *C. R. Acad. Sci. Paris Life Sciences*, **317**: 392-398.

Ye, F., Laigret, F., Whitley, J. C., Citti, C., Finch, L. R., Carle, P., Renaudin, J., and Bové, J.-M. (1992). "A physical and genetic map of the *Spiroplasma citri* genome." *Nucleic Acids Res.*, **20**: 1559-1565.

Ye, F., Renoudin, J., Bové, J.-M., and Laigret, F. (1994b). "Cloning and sequencing of the replication origin (*oriC*) *Spiroplasma citri* and construction of autonomously replicating artificial plasmids." *Curr. Microbiol.*, **29**: 23-29.

Young, T. F., Erickson, B. Z., Ross, R. F., and Wannemuehler, Y. (1989). "Hemagglutination and hemagglutination inhibition of turkey red blood cells with *Mycoplasma hyopneumoniae*." *Am. J. Vet. Res.*, **50**: 1052-1055.

Zhang, Q., Young, T. F., and Ross, R. F. (1994). "Glycolipid receptors for attachment of *Mycoplasmas hyopneumoniae* to porcine respiratory ciliated cells." *Infect. Immun.*, **62**: 4367-4373.

Zhang, Q., Young, T. F., and Ross, R. F. (1995). "Identification and characterization of a *Mycoplasma hyopneumoniae* adhesin." *Inf. Imm.*, **63**: 1013-1019.

Zhen, L., and Swank, R. T. (1993). "A simple and high-yield method for recovering DNA from agarose gels." *Biotechniques*, **14**: 894-898.

Zheng, J., and McIntosh, M. A. (1995). "Characterization of IS*1221* from *Mycoplasma hyorhinis*: expression of its putative transposase in *Escherichia coli* incorporates a ribosomal frameshift mechanism." *Mol. Microbiol.*, **16**: 669-685.

Zielinski, G. C., Young, T., Ross, R. F., and Rosenbusch, R. F. (1990). "Adherence of *Mycoplasma hyopneumoniae* to cell culture monolayers." *Am. J. Vet. Res.*, **51**: 339-343.

Zuckerkandl, E., and Pauling, L. (1965). "Molecules as documents of evolutionary history." *J. Theor. Biol.*, **8**: 357-366.